THE PATHOGENICITY OF AUJESZKY'S DISEASE VIRUS FOR EXPERIMENTALLY INFECTED CHICKENS, MICE AND RATS

By

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ABSTRACT OF THESIS

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Title of Thesis "The pathogenicity of Aujeszky's disease virus for experimentally infected chickens, mice and rats."

Aujeszky's disease virus was pathogenic for developing fowl embryos, young chicks, mice and rats. It induced cytopathic effects on kidney cell cultures derived from different species of mammals, on fibroblasts from fowl embryos and on kidney cells prepared from chickens of different ages.

In experimentally infected chickens, the mean duration of clinical disease was significantly influenced by the route of infection; the intracerebral route being the most effective. Chickens infected intranasally, intraocularly or intradermally survived longer than those infected intramuscularly or subcutaneously. In general, the mortality pattern was significantly related to the age of the birds at the time of infection, the route of infection and the dose of virus injected. The results of experiments in chickens of different ages infected intracerebrally, intramuscularly or subcutaneously with different doses of either the McFerran, Hungarian or Weybridge strain of virus, clearly showed that the relationship between the percentage of deaths and the age at the time of infection was inverse, linear or curvilinear and significant, and that the influence of age on the mortalities was independent of the dose of virus and the route of infection.

Deaths in chickens due to the introduction of Aujeszky's virus by different peripheral routes were always associated with the presence of virus in the brain. The virus titres were influenced neither by the age of the chickens at the time of infection nor by the dose of virus injected; but the rate of replication of the virus in the brain was related to the age at which the chickens were infected. Likewise, the mean duration of viraemia in the infected chickens was inversely related to age. Virus distribution in the different tissues of experimentally infected chickens depended on the strain of virus used. Intracerebral or intramuscular infection of chicks less than 24 hours old with the Hungarian strain of virus resulted in virus dissemination in the heart, lung, liver, spleen and kidney as well as in the brain and spinal cord. On the other hand, in chicks of similar ages infected with the McFerran strain, the virus was rarely detected in these tissues although, in intramuscularly infected birds, the virus was frequently present in the muscle tissue at the sites of inoculation and in the spinal cord.

In chickens surviving intracerebral, intramuscular or subcutaneous infection with Aujeszky's virus, resistance to intracerebral challenge infection was not conditioned by the age of the birds at the time of the first inoculation but was directly and significantly related to the amount of virus contained in the primary inocula. Specific resistance to intracerebral challenge developed as early as the fourth day after primary infection. Recovery from experimental infection was accompanied by the development of humoral virus neutralising antibodies, the titres of which were significantly higher in chickens receiving two or more doses of live virus than in those inoculated with inactivated virus.

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In general, the symptoms in infected rodents were of a varied nature but were more pronounced in rats than in mice. The chief clinical hallmark of experimental Aujeszky's infection in both species was hyperaesthesia of the skin. In infected mice and rats, the mean incubation periods ranged from 28 to 83 hours, the shortest being that induced by intracerebral infection. Rats and mice were equally susceptible to experimental Aujeszky's virus infection.

Virus was present in the brain of all mice and rats dying of experimental Aujeszky's disease, but there were differences in the distribution of virus in the parenchymatous organs. These differences were related to the routes of infection. As with chickens, exposure of rats and mice to Aujeszky's virus resulted in some resistance to challenge infection, but the degree of resistance was not absolute.

Complement-fixing and immunodiffusion antigens were demonstrable in infected cell cultures but only the former were detectable in the tissues of infected chickens and rats.

The work presented in this thesis is my own; and some aspects of the experimental findings have been published in co-authorship with one of my supervisors, Dr. G. Fraser.

Sakkubai P. Ramachandran

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SUMMARY

Aujeszky's disease virus was pathogenic for developing fowl embryos, young chicks, mice and rats. It induced cytopathic effects on kidney cell cultures derived from different species of mammals, on fibroblasts from fowl embryos and kidney cells prepared from chickens of different ages.

In experimentally infected chickens, the mean duration of clinical disease was significantly influenced by the route of infection; the intracerebral route being the most effective. Chickens infected intranasally, intraocularly or intradermally survived longer than those infected intramuscularly or subcutaneously. In general, the mortality pattern was significantly related to the age of the birds at the time of infection, the route of infection and the dose of virus injected. The results of experiments in chickens of different ages infected intracerebrally, intramuscularly or subcutaneously with different doses of either the McFerran, Hungarian or Weybridge strain of virus, clearly showed that the relationship between the percentage of deaths and the age at the time of infection was inverse, linear or curvilinear and significant, and that the influence of age on the mortalities was independent of the dose of virus and the route of infection.

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In chickens surviving intracerebral, intramuscular or subcutaneous infection with Aujeszky's virus, resistance to intracerebral challenge infection was not conditioned by the age of the birds at the time of the first inoculation but was directly and significantly related to the amount of virus contained in the primary

inocula. Specific resistance to intracerebral challenge developed as early as the fourth day after primary infection. Recovery from experimental infection was accompanied by the development of humoral virus-neutralising antibodies, the titres of which were significantly higher in chickens receiving two or more doses of live virus than in those inoculated with inactivated virus.

In general, the symptoms in infected rodents were of a varied nature but were more pronounced in rats than in mice. The chief clinical hallmark of experimental Aujeszky's infection in both species was hyperaesthesia of the skin. In infected mice and rats, the mean incubation periods ranged from 28 to 83 hours, the shortest being that induced by intracerebral infection. Rats and mice were equally susceptible to experimental Aujeszky's virus infection.

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Complement-fixing and immunodiffusion antigens were demonstrable in infected cell cultures but only the former were detectable in the tissues of infected chickens and rats.

REVIEW OF THE LITERATURE

GENERAL INTRODUCTION

Aujeszky's disease is an economically important disease in some regions of the world where there has been intensification of pig-rearing in recent years. Whereas the disease has been well controlled in the United States of America in the past decade, the epizootiological pattern of Aujeszky's disease in Europe is disquieting. Sporadic outbreaks continue to occur in Bulgaria, Czechoslovakia, Denmark, France, Hungary, Italy, Netherlands, Poland, Portugal, Rumania and Yugoslavia, suggesting a drift to endemicity, (Grunert and Skoda, 1964; F.A.O. Reports 1964, 1965, 1966, 1967, 1968); and the detection of virus-neutralising antibodies in a high percentage of apparently healthy pigs in some of these countries supports this hypothesis (Akkermans, 1963, Bendixen and Borgen, 1965; Borgen and Bendixen, 1965). The potential danger of Aujeszky's disease to the economy of these agricultural communities is readily appreciated when it is realised that the estimated swine population in these countries is as high as 70 millions (F.A.O. Report, 1968). It is also apparent that substantial numbers of these animals are at risk, because recent reports have indicated not only a gradual increase in the incidence of the disease but also an extension of the infection into territories which were hitherto free from the disease (Bendixen,

Bendixen and Christensen, 1965; Obel, 1965; Zuffa, 1965; Lucas, Metianu and Atanasiu, 1966).

The magnitude of the problem in Europe is highlighted by the fact that in Poland, which had remained free from the disease for a few years, a number of new outbreaks occurred in 1958, in fur-bearing animals (Ugorski, 1958). Since then, the disease has reappeared in farm livestock and outbreaks have been reported periodically in different parts of the country (Janowski and Oberfeld, 1965).

The disease is not so important in the United Kingdom, as it is in Europe, although it is occasionally encountered in Northern Ireland.

The causal agent of Aujeszky's disease is a member of the herpes group of viruses (Andrewes, 1962) and, as such, it shares several important biological features with other herpesviruses. These include the propensity to induce inapparent, latent infections in its principal natural host, namely the pig and the tendency for focal cytopathogenicity in cell cultures and fowl embryos, as well as the ability to replicate in the cell nucleus with the development of the characteristic nuclear inclusion bodies.

The virus has a broad spectrum of pathogenicity for different species of domesticated and free-living

mammals, and for some avian species but its ecological relationship with the pig is intimate and presents interesting epizootiological features. Aujeszky's disease behaves as a porcine analog of human and simian herpes in as much as a harmonious *modus vivendi* exists between the adult pig and the virus and the infection is transmitted to the young as soon as the passively-derived immunity declines to low levels (Burnet and Williams, 1939). Moreover, the pathological features of bovine pseudorabies are very similar to those of *herpes simiae* infection in man; and both diseases are almost always fatal. In addition to the striking similarities in their ecological behaviour and many of their epidemiological and pathological features, the demonstration of common antigens in the viruses of Aujeszky's disease, *herpes simplex* and *herpes simiae* (Burnet, Lush and Jackson, 1939; Plummer, 1964; Tokumaru and Scott, 1964) has strengthened the speculation that the three viruses have evolutionary links (Burnet, 1960).

HISTORY

Aujeszky (1902) is credited with the first description in the scientific literature of the disease which now bears his name and which is also referred to as pseudorabies. Nevertheless, there is some evidence that the malady had existed for several decades before this in parts of Western and Central Europe. The paucity of reliable accounts of the disease in the earlier literature is probably due to the quiescent and insidious nature of the disease in adult pigs, which rendered diagnosis difficult, together with the furious manifestations of the illness in cattle and sheep which were possibly mistaken for rabies.

However, towards the end of the last century, the astute clinical observations of Strebel (1889) and Bass (1910) provided a clue to the existence of the disease in Central Europe from about 1850 onwards. Likewise, the reports of Hart (1938), Harvey and Reid (1938), M'Corry (1938) and Stewart (1938) suggested that the disease had possibly occurred sporadically in Great Britain from the beginning of the twentieth century.

On the other hand, there seems little doubt that Aujeszky's disease existed in the U.S.A. by this time since the early American farm literature contains a number of remarkably detailed accounts of the condition described as "mad-itch". Hildreth (1824), the Ohio

physician, recognised the condition in cattle in several dairy farms in Marietta in 1813, and drew attention to the fact that dogs contracted the infection by feeding on infected beef. During the same period, a similar disease was reported in cattle in Talbot (Anon, 1823), Kentucky and Montgomery (S.B.D., 1823), and Tennesse (Hardeman, 1823). These observations were substantiated by several reports of the disease in cattle that appeared in the columns of "The Cultivator" (Demy, 1839), "The New England Farmer" (Anon, 1844), "Wisconsin and Iowa Farmer" (Morgan, 1855), and "American Agriculturist" (English, 1858; Hartzell, 1858). Moreover, two of the seven books dealing with livestock diseases that were published in America between 1840 and 1900 referred to "mad-itch" of cattle as a specific, infectious entity (Cole, 1847; McIntosh, 1895). The clinical and pathological features, particularly the short incubation period, the symptoms of pruritus, the fatal course and the transmissibility of the infection to dogs, bear a striking similarity to the later accounts of Aujeszky's disease. It is also interesting to note that in these early days the American workers fully appreciated the significance of the association between cattle and swine as a factor of prime epizootiological importance (Hanson, 1954).

Following Aujeszky's report of the disease in cattle dogs and cats in Hungary in 1902, the disease was recognised in other parts of Europe including Russia (Isabolinsky and Patzewitsch, 1912), Rumania (Riegler and Udrisky, 1914 and Riegler and Poenaru, 1920 cited by Jonnesco, 1934), Denmark (Bang, 1932), Holland (Burggraaf and Lourens, 1932), Yugoslavia (Nicolic, 1932), France (Rossi and Colin, 1934), Austria (Gerlach and Schweinburg, 1935) and Spain (Steiner and Lopez, 1935). The isolates from these outbreaks were confirmed as Aujeszky's disease virus by the experimental inoculation of rabbits (Galloway, 1938).

In the British Isles, Aujeszky's disease virus was first isolated and identified from three clinically affected bovines on a farm in Ulster (Lamont and Kerr, 1939) and, later in the same year, the disease was confirmed in pigs and cats (Lamont and Shanks, 1939). Subsequent reports of the disease in pigs (Gordon and Luke, 1952 and 1955; Venn and Done, 1953; McErlean, 1960; Johnston, Wittrick, Roberts and Done, 1961; Mackay, Done and Burrows, 1962), in cattle (Johnston et al., 1961), in dogs (Cassells and Lamont, 1942; Lamont and Gordon, 1950; Mullaney and Murphy, 1962; Dow and McFerran, 1963; Dawson, Evans and Jack, 1967; Huck, Evans, and Hooper, Davies and Williams, 1969) and in a cat (Dow and

McFerran, 1963) together with the results of several serological surveys (Glover, 1938; Johnston et al., 1961; Mackay et al., 1962; Burrows, 1963, 1966 and McFerran, Dow and Hildebrand, 1966) indicated that the disease was sporadic in nature in the United Kingdom.

In Brazil, the disease was reported by Carini and Maciel (1912), in an epizootic form affecting large heads of cattle, horses, mules and dogs. This and later outbreaks of the disease were clearly distinguished from vampire-transmitted rabies which was enzootic in that region (Kraus, Gerlach and Schweinburg, 1926; Braga and Faria, 1932, 1934).

In their accounts of Aujeszky's disease in pigs, Ratz (1914) and Burggraaf and Lourens (1932) stressed the insidious nature and mild course of the infection, as well as its high morbidity and low mortality; but it was Koves and Hirt (1934) who made pointed reference to the contagiousness of the porcine disease. The Brazilian (Patto, 1932 cited by Shope, 1935a), Dutch (Burggraaf and Lourens, 1932) and Hungarian investigators (Marcis, 1933; Koves, 1935) also observed that in all outbreaks involving cattle and sheep the animals had either been kept on premises previously occupied by pigs or had been in direct contact with pigs. The possibility that bovine pseudorabies infection generally originates from

a porcine reservoir was largely confirmed by the results of two detailed serological investigations by Shope (1935a, 1935b). Moreover, in an earlier report, Shope (1931) established unequivocally that the "mad-itch" of Iowa cattle was the same as Aujeszky's disease in Europe and that the two causative agents were immunologically identical.

Aujeszky's disease does not have a world-wide distribution, and apart from scattered reports of the disease in China (Lin, 1947; Lieou and Kono, 1950) in dogs in Algiers and Tunis (Remlinger and Bailly, 1931; Cordier and Menager, 1937) and in a pig in Angola (Mendes, Da Graca, Velho and Daskalos, 1954) there seems little doubt from the available evidence that pseudorabies is almost entirely confined to parts of Europe and the Americas (F.A.O. Reports 1964, 1965, 1966, 1967, 1968). The early history of the disease was reviewed <u>in extenso</u> by Koves (1935), Lourens (1935), Galloway (1938), Remlinger and Bailly (1938) and Hanson (1954).

THE VIRUS

The filterable nature of the agent of Aujeszky's disease was first established by Schmiedhoffer (1910) but Sangiorgi (1914) had only limited success in obtaining infective filtrates by means of Berkefeld N and Y filters. More encouraging results were obtained by Shope (1931), Koves and Hirt (1934), Koves (1935) and Remlinger and Bailly (1935) who clarified broth suspensions of infective tissues by a preliminary centrifugation before passing them through filter candles. Elford and Galloway (1936) used gradocol membrane filters of graded porosities and estimated that the particle size of the Hungarian and American strains of the virus was between 100 and 150 nm in diameter. This estimate is remarkably close to that of 90-100 nm suggested by Reagan, Schenek, Harmon and Brueckner (1952c) from their electron microscopic observations of the virus.

A significant landmark in the history of pseudorabies was the *in vitro* cultivation of the agent by Traub (1933). Following the methods used by Andrewes (1929a, 1929b and 1930) for the propagation of virus III and *herpes simplex* virus, Traub obtained good yields of the Hungarian strain of Aujeszky's disease virus in suspension cultures of minced rabbit and guinea pig testicular tissues and chick embryo tissues. Histological examination of the infected tissue fragments revealed necrosis of testicular

epithelial cells and the presence of eosinophilic, intranuclear inclusions in the vascular endothelial cells, fibroblasts and the interstitial cells of Leydig, but the pathogenicity of the virus was not altered after 49 serial passages.

Mesrobeanu (1938) cultivated the virus in fowl embryos, and his findings were soon confirmed by other workers notably Glover (1939), Burnet et al (1939) and Bang (1942). In their report, Burnet and his colleagues also noted that the replication of the virus on the chorio-allantoic membranes was associated with the production of circumscribed, grayish or whitish, necrotic, focal lesions histologically reminiscent of the 'plaques' formed by herpes simplex and herpes simiae. Moreover, Bang (1942) and Sabban (1949) observed that the ability to invade the central nervous system and to produce a haemorrhagic encephalitis in the embryo was a reproducible characteristic of Aujeszky's disease virus. Other workers found that the tendency for focal cytopathogenicity was manifest when the virus was inoculated into monolayer cultures of fowl embryo cells (Beladi and Szollosy, 1955) and that the virus showed a wide spectrum of pathogenicity for primary cultures and cell lines derived from different mammalian species (Kaplan, 1966, Lautie, 1969).

The next exciting development in the characterisation studies of Aujeszky's disease virus was the visualization of the intact particles of virus by electron microscopy (Kaplan and Vatter, 1959). Electron micrographs of thin sections of virus pellets embedded in methacrylate revealed spherical particles containing an electron dense core and a limiting double membrane. Morphologically the particles were very similar to those of herpes simplex (Morgan, Ellison, Rose and Moore, 1954) and herpes simiae (Reissig and Melnick, 1955). Indirect evidence suggesting that the electron dense core was the site of the viral DNA emerged from the studies of Reissig and Kaplan (1962) in which 5-fluorouracil was incorporated in the medium to block the synthesis of the host cell DNA. No viral DNA was synthesised but incomplete virions lacking an electron dense core were produced (Reissig and Kaplan, 1962). Direct incontrovertible evidence that the nucleic acid of pseudorabies virus was DNA was provided by the studies of Ben-Porat and Kaplan (1962). They centrifuged the virus labelled with p32 in suspensions of cesium chloride to establish an equilibrium density gradient and observed that the peak of radioactivity associated with the DNA fraction coincided with the peak of infectivity. Moreover, comparative studies with herpes simplex virus

showed that the DNA of the two viruses had a similar base composition with a relatively high proportion of guanine and cytosine (Kaplan and Ben-Porat, 1964). The results of heat and formaldehyde denaturation studies suggested that the DNA of pseudorabies virus was double-stranded and with a molecular weight of 68 to 70 x 10^6 daltons (Kaplan and Ben-Porat, 1964).

As with many other animal viruses recent research on Aujeszky's disease virus has been mostly directed towards the attenuation of the virus in heterologous hosts and cell cultures with the objective of evolving satisfactory virus vaccines and, in this respect, the Balkan, Russian and Central European workers have given a significant lead. Trials with chemically inactivated virus suspensions in domestic livestock, mink and foxes were reported to be successful (Ribarov, Boyadzhiev and Draganov, 1960; Lyubashenko and Tyulpanova, 1961; Nedyalkov, Ribarov, Stoychev and Stoylova, 1968), but the immunising efficiencies of the preparations are difficult to assess because either the vaccine trials were conducted on small scales (Ribarov et al., 1960) or details of the tests were not given (Lyubashenko and Tyulpanova, 1961 cited by Pool, 1964). However, the results of trials in pigs and cattle with pigeon-passaged (Toneva, 1961) or fowl embryo cell-cultured (Skoda, Brauner, Sadecky and

Somogyiova, 1962) or fowl embryo-adapted virus strains (Bran, Suhaci and Ursache, 1965) have been encouraging.

Latency in the natural host is an established biological characteristic of Aujeszky's disease virus. Hence the use of live virus vaccines for prophylaxis must be viewed with caution until such time as the behaviour of the "attenuated" virus strains in the species of animals at risk has been fully elucidated.

PATHOGENICITY

NATURAL PATHOGENICITY

General: The list of domestic, captive and free-living mammals implicated in Aujeszky's disease includes several genera in the orders Lagomorpha, Rodentia, Carnivora and Artiodactyla (Tables I and II). Of these, outbreaks of the natural disease have been proven in the pig, ox, sheep, dog, cat, mink, ferret, free-living and captive arctic fox, silver-backed fox, red fox, field rat and gray field mouse (Table I). Natural infections have also been reported in a restricted number of captive and free-living mammals (Table II); but the allegations were not supported by experimental data.

Rat: The rat is often incriminated in the natural disease (Balas, 1908; Schmiedhoffer, 1910; Grunert and Skoda, 1964) and most early reports present compelling circumstantial evidence that they play an important role in the spread of the infection. This was well illustrated in the reports of Balas (1908), Hutyra (1910) Burggraaf and Lourens (1932), Gordon and Luke (1952), Lyubashenko, Tyulpanova and Grishin (1958) and Kretzchmar (1964) who remarked on the large numbers of dead rats found on infected pig and cattle farms. Remlinger, Bailly and Rossi (1933) also noted that cases of the disease in farm dogs and cats were generally preceded by an invasion of rats while Bartosz (1962)

TABLE 2

ALLEGED NATURAL INFECTIONS OF DOMESTIC, CAPTIVE AND FREE-LIVING MAMMALS WITH AUJESZKY'S DISEASE VIRUS

Genus	Common name	Original reference	Supporting reference
Lepus	Hare	Grunert and Skoda (1964)	-
Sciurus	Squirrel	Mikolitsch (1954)	-
Ursus	Bear	Arsov and Jovcev (1958)	-
Procyon	Racoon	Ihlenberg and Senf (1957)	-
Meles	Badger	Ratz (1913)	Lourens (1935)
Lutra	Otter	Grunert and Skoda (1964)	-
Capreolus	Roe deer	Snajber and Kunjakov (1956)	-
Capra	Goat	Zwick and Zellar (1911)	Burggraaf and Lourens (1932)

observed debilitated and cachectic rats on infected farms. Lamont and Kerr (1939) and Janowski and Oberfeld (1965) observed that rats disappeared from farms a short time before or in the course of outbreaks of pseudorabies in pigs implying that the exodus of rats was the result of deaths from the disease. It is also of interest that an outbreak in ferrets was attributed to the use of these animals for the eradication of rats from infected piggeries (Ratalics, 1965).

There are few authenticated reports of the isolation of Aujeszky's disease virus from naturally infected rats (Hutyra, 1910; Tepper, 1957; Nikitin, 1960; Becker and Herrmann, 1963) or indeed from other species of rodents (Lukashev and Rotov, 1939); but the most complete is that of Nikitin who isolated the virus from the brain, lungs and urinary bladder of rats that were trapped on a farm about 2 months after the cessation of an outbreak of Aujeszky's disease in pigs. The fact that the virus isolations were made after the animals were observed for a period of about 10 weeks, is excellent proof of the carrier state in this species. Nikitin also gives details of a massive outbreak in rats which was responsible for the deaths of about 1,500 animals, the high mortality being followed 10 days later by clinical disease in pigs. Two months after the outbreak in pigs,

11 apparently healthy adult rats were trapped on the farm and, after an observation period of about 2 months, were examined for virus. In each case, Aujeszky's disease virus was isolated from the lungs, urinary bladder and urine. On the other hand, Lamont (1947) failed to recover the virus from pooled brain suspensions of 42 wild rats (Mus. decumans) trapped on infected farms and knackeries in Northern Ireland and examination of the sera of 15 of the rats revealed no neutralising antibodies to Aujeszky's virus. Likewise, the efforts of Aldasy and Mate (1969) to isolate the virus from 1078 organ samples of 298 rats (Rattus norvegicus) were unsuccessful.

Horse: Reports of the natural disease in horses are mostly based on the observation of suspicious symptoms in horses kept on the same premises in which clinical disease had occurred in pigs (Burggraaf and Lourens, 1932; Koves and Hirt, 1934; Finkelstein, 1940). In some investigations, a number of horses are reported to have died after showing pronounced symptoms of central nervous involvement (Panisset, 1935; Lukashev and Rotov, 1939) but none of the reports make it clear whether the virus was actually recovered. Moreover, statements that the virus was isolated from allegedly infected horses (Eidson, Kissling and Tierkel, 1953; Innes and Saunders,

1962) are not supported by virological data.

EXPERIMENTAL PATHOGENICITY

Natural Hosts: In most natural hosts, the disease is easily reproduced under experimental conditions, but the susceptibility of the pig to parenteral infection merits particular emphasis.

Pig: In the early investigations some workers (Schmiedhoffer, 1910; Lamont and Shanks, 1939; Lamont, 1946) failed to induce the disease in pigs while others reported success (Shope, 1935a; Andrev and Uzunov, 1940; Hirt, 1942; Shahan, Knudson, Seibold and Dale, 1947a), but these discrepancies were probably due either to the use of suspensions of infective materials of undetermined virus activity or to the degrees of innate resistance of the pigs that were used in the studies. There is, moreover, a marked age-linked susceptibility to experimental infection in the pig (Hirt, 1935; Vianello, 1942; Shahan et al., 1947a; Akkermans, 1963; Olander, Saunders, Gustafson and Jones, 1966; Csontos, 1969) and even in fully susceptible age groups, introduction of virus by most parenteral routes does not always result in overt disease. Also, in many instances, inapparent infection may ensue (Lukashev and Rotov, 1939; Kojnok, 1962; Shope, 1964). Intracerebral infection is

uniformly fatal (Hurst, 1933; Lamont and Shanks, 1939; Hirt, 1942; Shahan et al., 1947a) whereas intranasal infection, although not generally fatal, usually results in high success rates and is the method mostly preferred in studies of the pathogenesis of the disease, (Lukashev and Rotov, 1939; Taga, Berbinschi, Cirstet and Coman, 1957; McFerran and Dow, 1964c,1965). There is no evidence that intracerebral passage of the virus in pigs causes either attenuation or exaltation of virulence for this species (Hirt, 1942).

Ox: Cattle can readily be infected with Aujeszky's disease virus (Bang, 1932; Burggraaf and Lourens, 1932; Jansen and Kunst, 1957; McFerran and Dow, 1964a) and mortality rates of 100 per cent or thereby have been reported by McFerran and Dow (1964a) and by Braga and Faria (1932 and 1934). All parenteral routes are effective in inducing the clinical disease (McFerran and Dow, 1964a). However, according to some investigators intranasal infection is not reliable (Lamont, 1946) and sometimes fails to produce disease (Masic, 1961). A significant feature of the experimental disease in cattle is that the infection does not spread by contact (Shahan et al., 1947a) and is, therefore, reminiscent of the natural disease in this species (Aujeszky, 1902; Zwick and Zeller, 1911; Shope, 1931).

Sheep: There are relatively few accounts of experimentally induced Aujeszky's disease in sheep (Schmiedhoffer, 1910; Ratz, 1914; Burggraaf and Lourens, 1932; Koves, 1935; Salyi, 1935; Hirt, 1936) but it seems from the evidence that is available that sheep are just as susceptible to the infection as cattle are. By using a significant number of animals, Dow and McFerran (1964) noted that the experimental disease in 6-12 months' old Black-face lambs was always fatal irrespective of the route of inoculation and similarly that a large number of mature Black-face sheep used in their virus titration studies succumbed to the infection. Animals that survived apparently did not receive any virus. Ivanov, Kharalambi and Stamenov (1968) induced the disease in 2 of 10 sheep by the conjunctival route, but failed to do so by the oral route of infection.

Cat and dog: Several papers have been published which suggest that cats are more susceptible than dogs to experimental pseudorabies (Remlinger and Bailly, 1934; Galloway, 1938; Andrev and Uzunov, 1940; Ercegovac, Trumic, Lapcevic and Ciric 1958) and in one of these reports (Remlinger and Bailly, 1934) it was claimed that virus of bovine origin inoculated into rabbits, guinea pigs, rats, a dog and a cat produced clinical symptoms of the disease only in the cat. But, unfortunately,

parallel titrations were not undertaken in the dog and cat to test this hypothesis.

Cats and dogs are readily infected *per os* and by other parenteral routes (Zwick and Zeller, 1911; Remlinger and Bailly, 1933c; Andrev and Uzunov, 1940; Ercegovac *et al.*, 1958).

The comparative susceptibilities of rabbits, guinea pigs, white mice and dogs to Aujeszky's disease was investigated by Jonnesco (1948) using a strain of reduced virulence isolated from a cat. From the results of this experiment Jonnesco concluded that the mean incubation period was shortest in rabbits and longest in dogs.

Ferret and mink: Ferrets (Goret and Mariett, 1938; Goto, Gorham and Burger, 1967; Goto, Gorham and Hagen, 1968) and mink (Goto *et al.*, 1968) are highly susceptible to experimental infection with the European and American strains of the virus and the susceptibilities of the two species to subcutaneous infection with the Shope and BUK (Skoda, 1962) strains of the virus have been rated as high as that of the rabbit (Goto *et al.*, 1968). Goto and his colleagues also demonstrated that ferrets were readily infected by the aerosol method and that the survivors did not resist reinfection with 100 ferret infective doses.

Rat: Aujeszky's disease is known to be infective to rats under experimental conditions but quantitative data are lacking regarding the susceptibilities of wild and laboratory-bred rats of different ages and breeds. While Shope (1931) observed that white rats were regularly infected by the intracerebral route, the pattern of deaths following intraperitoneal inoculation was irregular and most resisted subcutaneous infection. In general, wild brown rats (Mus norvegicus) are less susceptible than white rats, and the percentage of deaths is uniformly low (Shope, 1935b). According to Burggraaf and Lourens (1932) and Lourens (1935) rats could not be infected by the subcutaneous, intraperitoneal or oral routes, although they invariably succumbed to intracerebral inoculation. Likewise, Andrev and Uzunov (1940) reported that they were able to infect white rats by the intraocular route only. In this connection it is interesting to note that a possible relationship between the ages of rats and their susceptibilities to experimental Aujeszky's disease emerged from the studies of Koch and Abraham (1952) who found that rats above 6 months of age could be infected only by the intracerebral route.

Although rats can readily be infected by feeding, a number of workers have observed variations in the

susceptibilities of different species (Hutyra, 1910; Schmiedhoffer, 1910; Zwick and Zeller, 1911; Bang, 1932; Remlinger and Bailly, 1934; Gerlach and Schweinburg, 1936; Lamont, 1947). For instance, Shope (1935b) noted that white rats were more readily infected by feeding on infected carcases than were wild brown rats, while Andrev and Uzunov (1940) reported that white rats were relatively less susceptible than gray rats. McFerran and Dow (1970) used a large number of wild and laboratory rats in their studies and provided quantitative data on the relative susceptibilities of these animals to infection. The strain of virus, which had an infectivity titre of $10^{7.0}$ for pig kidney cells, was infective to the laboratory strain of rats up to dilutions of 10^{-4} but only if given by the intranasal route. The oral route was barely infective. They also noted that the wild rat was less sensitive than the laboratory rat except when infected per os.

The experimental disease in rats is non-contagious (Shope, 1935b) and rats do not seem to spread the infection through bites to other rats (McFerran and Dow, 1970) or to other species of animals (Remlinger and Bailly, 1937).

Horse: The behaviour of the virus in horses is enigmatic. Hutyra, Marek and Manninger (1926) and Koves (1939)

regarded this species as being highly resistant to experimental infection and Braga and Faria (1934) failed to infect six horses and three mules when large doses of virus were given by different parenteral routes. On the other hand, Schmiedhoffer (1910) was successful in inducing clinical symptoms in 4 out of 6 horses. Three of the four affected horses died but it is not clear whether the virus was isolated from the dead animals. In the studies of Panisset (1935) 50 per cent of the infected horses died showing marked nervous symptoms. Similarly, 6 of 10 horses infected by Popescu (1966) developed febrile and nervous symptoms and four of them died. These investigators did not report virus recovery.

Gerlach and Schweinburg (1935) noted paralytic symptoms in one of three horses inoculated with a suspension from an entire infective rabbit brain but it is arguable whether or not the symptoms were induced by the virus since no transmission studies were attempted with materials from this animal. Lamont (1946) failed to induce clinical symptoms by the inoculation of 5 horses subcutaneously with 100-250 guinea pig minimal lethal doses (MLD) of rabbit brain virus and of two others <u>per os</u> with 400 guinea pig MLD of the same material. Nor was he able to demonstrate neutralising antibodies in their sera. In a second trial one of 2 horses inoculated subcutan-

eously with the virus developed nervous signs on the 13th day but blood collected a day later, proved to be non-infectious to a rabbit. The second horse which received several subcutaneous inoculations and was also fed massive amounts of the virus did not develop symptoms. However, in spite of these irregular results, neutralising antibodies were demonstrated in the sera of both animals.

Experimental Hosts:

The spectrum of experimental pathogenicity of pseudorabies virus embraces several species of mammals in the orders Marsupialia, Insectivora, Chiroptera, Primates, Lagomorpha, Rodentia, Carnivora and Artiadactyla (Table 3) and nine species of birds (Table 4).

Rabbit: Until the introduction of cell culture techniques the rabbit was generally regarded as being the most suitable experimental host for the isolation of Aujeszky's disease virus. (Gerlach and Schweinburg,1936; Galloway, 1938; Jonnesco, 1948) While McFerran and Dow (1962) did not find any significant differences between the susceptibilities of pig kidney cell cultures and rabbits inoculated by the subcutaneous and the intra-cerebral routes, Selevanov, Khasanov and Kamalov (1970) reported that rabbits were more sensitive to aerosol infection than fowl embryo fibroblast cultures. Bang

TABLE 3

MAMMALS EXPERIMENTALLY INFECTED WITH AUJESZKY'S DISEASE VIRUS

Genus	Common name	Original reference	Supporting reference
Order Marsupialia			
Didelphis	Opposum	Braga and Faria (1934)	Trainer and Karstad (1963)
Order Insectivora			
Erinaceus	Hedgehog	Aujeszky (1902)	Remlinger and Bailly (1933f)
Order Chiroptera			
Eptesicus	Brown bat	Reagan *et al* (1953)	-
Order Primates			
Calithrix	Marmoset	Braga and Faria (1934)	-
Macacus	Rhesus monkey	Hurst (1934)	Reagan *et al* (1952b)
Order Lagomorpha			
Lepus	European rabbit	Aujeszky (1902)	Schmiedhoffer (1910)
Sylvilagus	Cotton tail rabbit	Trainer and Karstad (1963)	-

TABLE 3 contd.

Genus	Common name	Original reference	Supporting reference
Order Rodentia			
Mormota	Wood chuck	Trainer and Karstad (1963)	-
Crisetus	Hamster	Reagan *et al* (1952a)	Reagan *et al* (1956)
Ondatra	Muskrat	Trainer and Karstad (1963)	-
Hystrix	Porcupine	Braga and Faria (1934)	-
Cavia	Guinea pig	Aujeszky (1902)	Schmiedhoffer (1910)
Order Carnivora			
Canis	Jackal	Remlinger and Bailly (1933i)	Lourens (1935)
Mustela	Polecat	Lourens (1935)	-
Martes	Sable	Tyulpanova and Grabovskii (1964)	-
Mephitis	Skunk	Trainer and Karstad (1963)	-
Order Artiodactyla			
Odocoileus	Deer	Trainer and Karstad (1963)	-

TABLE 4

BIRDS EXPERIMENTALLY INFECTED WITH AUJESZKY'S VIRUS

Genus	Common name	Original reference	Supporting reference
Order Anseriformes			
Anas	Duck	Shope (1931)	Remlinger and Bailly (1933a)
Anser	Goose	Remlinger and Bailly (1933a)	-
Order Columbiformes			
Columba	Pigeon	Remlinger and Bailly (1933a)	Jonnesco (1934)
Order Falconiformes			
Accipter	Sparrow-hawk	Remlinger and Bailly (1933i)	-
Buteo	Buzzard	Remlinger and Bailly (1933a)	Lourens (1935)
Falco	Falcon	Lourens (1935)	-
Order Galliformes			
Gallus	Fowl	Shope (1931)	Remlinger and Bailly (1933a)
Meleagris	Turkey	Remlinger and Bailly (1939)	-
Order Passeriformes			
Serinus	Canary	Remlinger and Bailly (1933a)	-

(1942) found that strains of Aujeszky's disease virus that had been serially passaged in rabbits produced haemorrhagic lesions on the chorio-allantoic membranes and other tissues of fowl embryos more consistently than did other strains of the virus.

It is now well known from the detailed studies of Shope (1931), Remlinger and Bailly (1933b), Hurst (1934) and Andrev and Uzunov (1940) that most parenteral routes are equally effective in rabbits. However, it has also frequently been shown that the incubation period following intracerebral inoculation is considerably shorter than that following the intravenous, subcutaneous, intramuscular or intradermal routes (Shope, 1931; Remlinger and Bailly, 1933b; Ercegovac, 1960; McFerran and Dow, 1962; Goto, Gorham and Hagan, 1968). Symptoms following intracerebral inoculation may occur as early as 20 hours (Shope, 1931) but generally range from 20 to 60 hours (Shope, 1931; Hoyt, 1946). Unfortunately, however, most observations on the relative efficiencies of the different routes of inoculation are not based on quantitative data. Employing a rabbit passaged strain of the virus Shahan et al (1947b) observed that dilutions up to 10^{-6} were infective by the intracerebral route, while with a rabbit cell culture-adapted strain McFerran and Dow (1962) obtained titres of $10^{6.5}$ to $10^{6.75}$ by the

subcutaneous route of infection. Thus, it would seem that the subcutaneous route is just as effective as the intracerebral route in virus recovery studies.

Experimental infection was also established in rabbits by the oral (Remlinger and Bailly, 1933b) and intratesticular routes (Shope, 1931, Remlinger and Bailly, 1933b) and after administration of the virus into the anterior chamber of the eye (Remlinger and Bailly, 1938); but the intraconjunctival route generally failed (Remlinger and Bailly, 1933b; Morril and Graham, 1941).

The experimental disease does not appear to spread by contact, and Shope (1931) who induced the fatal infection in a pregnant rabbit doe, observed that the progeny did not contract the disease within two weeks of birth. Nor were rabbits infected through the bites of experimentally infected foxes (Nicolic, 1934) or cats, rats or hedgehogs (Remlinger and Bailly, 1933f, 1937).

There is also evidence that the state of metabolic activity of the animal may influence the pathogenesis of experimental Aujeszky's disease in the rabbit since artificially induced hibernation not only considerably delays the onset of the symptoms in infected rabbits (Jelesic, 1955) but also suppresses the development of virus in the brain (Jelesic, Kikinis-Jelesic and Jelesic,

1955). Similarly depression of the CNS by chloral hydrate prolongs the duration of the disease in experimentally infected rabbits (Kuzmin, 1959). However, these reports await confirmation.

Guinea pig: Guinea pigs are readily infected by various parenteral routes (Shope, 1931; Remlinger and Bailly, 1933b; Nicolau, Cruveilhier and Kopciowska, 1937a; Carneiro, 1940; Ercegovac et al., 1958) and by aerosol exposure (Selivanov et al., 1970).

Shope (1933) observed that the guinea pig was 100 times more resistant than the rabbit to experimental pseudorabies while Gerlach and Schweinburg (1936), who studied the pathogenicity of 8 strains of virus for rabbits and guinea pigs, noted that relatively larger doses of the virus were necessary to induce the disease in guinea pigs. Jonnesco (1948) reported that the incubation period in guinea pigs was longer than in rabbits when injected with the same dose of virus by the same route. On the other hand, Shahan et al (1947b) found no significant differences in the onset of symptoms, duration of the disease and the pattern of deaths when guinea pigs and rabbits were inoculated by the intracerebral route, but Shope (1931) reported that virus titres in the brains of intracerebrally infected guinea pigs were at least 10-fold less than the titres in

the brains of similarly infected rabbits. Moreover, Shope (1933) observed that following several serial intracerebral passages in guinea pigs the passaged virus failed to infect the same species of animal subcutaneously except in "massive" doses. However, its pathogenicity for the guinea pig by the subcutaneous route was restored by a single passage in the rabbit.

Mouse: There is general agreement that laboratory-bred mice are less susceptible than guinea pigs to experimental pseudorabies (Shope, 1931; Remlinger and Bailly, 1933b; Shahan et al., 1947b; Jonnesco, 1948). This opinion stems from the early work of Aujeszky (1902) who failed to infect mice with virus recovered from naturally infected cattle and from a more recent review article by Galloway (1938) who regarded mice as being relatively refractory to experimental infection. Again, unfortunately, most workers do not give details regarding the ages and breeds of mice used in their studies. For instance, Shope (1931) reported that field mice were susceptible to pseudorabies virus but did not mention the age of the animals concerned.

Shahan et al (1947b) compared the sensitivities of the rabbit, guinea pig and the laboratory mouse to a rabbit passaged strain of pseudorabies virus. Whereas, no significant differences in species susceptibilities were detectable by intracerebral infection, larger doses

were needed to infect mice subcutaneously.

Whether or not mice can be infected per os probably depends on the concentration of virus in the materials. Some workers (Schmiedhoffer, 1910) were successful by this route while others (Burggraaf and Lourens, 1932) failed to infect mice by the oral, subcutaneous and intraperitoneal routes. Nutritional deficiences may also affect the pathogenesis of the disease in experimentally infected animals. For example, Toneva (1967) reported that hypovitaminosis A reduced the incubation period in experimentally infected mice. Not only were the clinical symptoms more pronounced in the animals but the death rates were also higher than in the control groups.

Hamster: Hamsters are also susceptible to experimental Aujeszky's disease and, according to Reagan, Yancey and Brueckner (1956) intraperitoneal inoculation of lactating hamsters results in spread of the infection to their unweaned offspring. However, in a second trial they showed that unweaned hamsters infected by a variety of routes, including the oral, rectal and intralingual routes invariably succumbed to the disease and that there was no clinical evidence of lateral spread of the infection to the mothers (Reagan et al., 1957).

Hedgehog: The hedgehog is also susceptible and Aujeszky

(cited by Galloway, 1938) and Remlinger and Bailly (1933f) and Lourens (1935) successfully infected hedgehogs by different routes. The presence of the virus was confirmed by subinoculation into rabbits.

Monkey: Different simian species that have been successfully infected include rhesus and marmoset monkeys (Braga and Faria, 1934; Hurst, 1936, 1936; Reagan, Harman, Day and Brueckner, 1952b). Using Bartha's (1961) attenuated strain of Aujeszky's disease virus Karasszon (1965) showed that monkeys could be infected by either the intracerebral or intraspinal routes. In the affected animals, localised inflammatory changes were detected in the temporal and basal regions of the brain, while the lesions in the spinal cord were generally localised near the site of inoculation. The nature of the histological changes suggested a degree of viral replication with a possible fatal termination had not the animals been killed on the 18th day post infection. Hurst (1936) noted that monkeys that were "immune" to virus B infection frequently resisted intracerebral inoculation with Aujeszky's virus whereas others which had no antibodies were susceptible. The virus was pathogenic by the intracerebral, intracisternal and intrasciatic routes but not by intradermal, intramuscular and intravenous routes (Hurst, 1936). The

failure to infect monkeys by other workers (Hurst, 1930; Remlinger and Bailly, 1934; Nicolau et al., 1937a) has been partly attributed to the presence of antibodies to herpes simiae.

Bat: Bats are susceptible to experimental infection with Aujeszky's disease virus (Lourens, 1935). In the United States, Reagan, Day, Marley and Brueckner (1953) infected the large brown bat (Eptesicus fuscus) by a variety of routes. Symptoms of central nervous involvement appeared in 72 to 96 hours and the virus was recovered from the brains of dead bats by inoculation into guinea pigs.

Man: There are reports in the early European literature of alleged but unproven human infections due to the agent of Aujeszky's disease (Ratz, 1914; Nicolic, 1932; Braga and Faria, 1934). On the other hand, Remlinger and Bailly (1934, 1935) believed that the virus was non-pathogenic for man, but they did not provide experimental data in favour of this hypothesis. Truche, Cruveilhier and Viala (1935) and Tuncman (1938) and Tuncman and Schukru-Aksel (1938) reported on what they considered was Aujeszky's disease in laboratory workers who had received accidental injuries to their hands while dealing with infective materials. Because one of Tuncman's patients (Tuncman, 1938) developed

intense localised pruritus within three hours of the injury, this was criticised by Galloway (1938) as being too short an incubation period for classical Aujeszky's disease. The second patient did not develope pruritus until 18 hours after the injury and the mild febrile symptoms persisted for 3 days. Blood from this patient, as well as from one of the 2 patients mentioned by Truche et al (1935) was infective to rabbits and/or guinea pigs but unfortunately, the identity of the agent was not confirmed serologically.

Bird: Little is known about the susceptibility of birds to the virus of Aujeszky's disease and the natural disease has not been reported so far in this species. Nor is there evidence that the infection can spread to poultry that have been in direct contact with infected pigs and cattle (Estola et al., 1965). Moreover, there are conflicting reports in the literature on the relative susceptibilities of different avian species to the experimental infection. Much of the confusion has arisen because earlier workers used infective suspensions containing different amounts of virus in birds of undetermined ages.

The report of Shope (1931) that ducks and hens were susceptible to intracerebral inoculation with the Iowa strain of pseudorabies virus was confirmed and extended

by Remlinger and Bailly (1933a, 1933i), who found that the same strain of the virus was also infective to pigeons, geese, buzzards and sparrow-hawks. Later, they demonstrated that turkeys were susceptible by the intracerebral route, whereas guinea fowls were highly resistant (Remlinger and Bailly, 1939). The susceptibility of pigeons to the experimental infection was confirmed by Jonnesco, 1934; Gerlach and Schweinburg, 1936; Nicolau et al., 1937a; Bailly, 1939; Koves, 1939 and Shahan et al., 1947b, some of whom consider that pigeons are more susceptible than poultry (Jonnesco, 1934; Nicolau et al., 1937a), since the incubation period in pigeons was shorter and the percentage of mortalities was higher. Furthermore, in the studies of Shahan et al., (1947b) pigeons that survived infection reacted clinically to intracerebral challenge with a higher dose of virus but only a few of these succumbed. Serial intracerebral passages of the virus in pigeons resulted in the enhancement of virulence for this species (Toneva, 1961) and the incubation period was reduced from 15 to 2 days after 30 passages. However, the pigeon-adapted virus did not induce clinical symptoms in rabbits and mice, although it immunised them against challenge infection with virulent virus.

Many workers consider that domestic poultry are

either resistant (Aujeszky, 1902; Schmiedhoffer, 1910; Zwick and Zeller, 1911; Hutyra et al., 1926; Braga and Faria, 1932) or only slightly susceptible to experimental inoculation with the virus of Aujeszky's disease (Nicolau et al., 1937a). However, Shope (1931), Remlinger and Bailly (1934), Gerlach and Schweinburg (1936) and Koves (1939) successfully infected chickens by the intracerebral route, although the mortality figures varied. In Shope's experiments two out of three chicks died on days 6 and 11 respectively and the presence of the virus was confirmed in the brain of the first chick by inoculation into rabbits. Nicolau et al (1937a) infected three hens intracerebrally. One died on the fifth day, the second was killed on the twelth day, when symptoms suggestive of central nervous involvement were well-marked, and the virus was isolated from its brain. The third bird did not show any overt clinical signs up to thirteen days, but necropsy revealed lesions of viral encephalomyelitis. Although these workers did not comment on the ages of their experimental birds, Bang (1942) observed that 50 per cent of 2 days' old chicks succumbed to the disease following intracerebral infection. Likewise, Shahan et al (1947a) noted that chicks of 12 to 13 days of age were highly susceptible to the virus when inoculated intracerebrally while Ivanovics, Abraham and Koch (1954) infected chicks of 1

to 16 days of age by the intracerebral and subcutaneous routes and recovered the virus from the brain tissue of birds in both groups.

Fowl embryo: Mesrobeanu (1938) was the first to cultivate the virus of Aujeszky's disease in the fowl embryo but his comments were mainly confined to the cellular responses in the infected chorio-allantois. Following this report, Badenski and Bruckner (1938), Glover (1939), Burnet et al (1939) and Bang (1942) demonstrated that there was also active multiplication of the virus in the chorio-allantoic membranes.

In the studies of Badenski and Bruckner (1938), the replicative phase was transient; virus titres declined from the second day post-inoculation and no virus was demonstrable in the infected membranes after six serial passages. Glover (1939) observed that replication of the virus in the chorio-allantois was followed by invasion of the embryonic tissues while Bang (1942) demonstrated that blood was infective and that the virus reached the brain via the blood stream.

It is interesting to note that while Suhaci, Ursache and Tomescu (1956) successfully infected fowl embryos by the intracerebral route they were unable to propagate the virus on the chorio-allantois until the virus had first undergone eight serial intracerebral

passages in nine-day old embryos. They also found that the virus titres in the embryonic brains were higher than those in the brains of experimentally infected rabbits. Others have reported that serial passage in fowl embryos results in alteration of the pathogenicity of the virus for rabbits (Glover, 1939; Burnet et al., 1939), guinea pigs (Glover, 1939) and mice (Burnet et al, 1939), which is characterised by a delay in the onset of symptoms, prolongation of the survival period and also diminution or even absence of pruritus. On the other hand, Suhaci et al (1956) did not obtain modification in virulence even after 30 serial passages in fowl embryos.

Serial passage in the embryos led to exaltation in virulence for this host and Glover (1939) noted that after fifty-five passages the virus was uniformly fatal to the chicken embryos and that deaths generally occurred on the 3rd day after infection, with well-marked haemorrhages in the skin. In the experiments of Burnet et al (1939) fatalities were rare in the early passages whereas they occurred in about 50 per cent of infected embryos in the later passages. Bang (1942) observed that infection with early passaged virus was characterised by the presence of large "pocks" only, whereas after serial passage the virus produced smaller "pocks" together with many "satellite pocks". He also observed that embryo-passaged virus readily induced

haemorrhagic encephalitis with destruction of the vascular endothelium. Freshly isolated strains of the virus did not show this property, although they did induce lesions of encephalitis.

In the same report Bang noted a correlation between the ages of the embryos and their susceptibility to pseudorabies infection. Twelve days' old embryos showed well-defined "pocks" on the chorio-allantoic membranes and extreme nerve cell destruction with marked haemorrhages in the brain, whereas, in 15 days' old embryos there were scattered "pocks" and diffuse encephalitis without haemorrhages. Eighteen days' old embryos did not show detectable lesions. Another factor which may influence the development of the virus on the chorio-allantois is the temperature of incubation. Thus, Ivanovics et al (1954) found that the number of "pocks" in infected eggs was fewer at 40°C than at 36°C. Unfortunately they did not comment on differences, if any, in the infectivity titres.

Cell Culture: The versatility of pseudorabies virus to adapt itself to experimental hosts is reflected in its in vitro pathogenicity for cells derived from chicken embryos and a wide range of mammalian species. The many susceptible cell culture systems include renal epithelial cells from calves (Bodin and Greczi, 1966; Bodon, Meszaros, Papp-Vid and Romvary, 1968; Matis and

Zuffa, 1969), dogs (Torlone, 1958; Baldelli and Torlone, 1958; Morozzi, 1959; Gagliardi, Borghi and Girotto, 1960), lambs (Ceccarelli and Delmazza[illegible], 1958; Gaverilov, 1960), mink (Rondhuis and Haagsma, 1966), monkeys (Tokumaru, 1957; Kersting, Kerekjarto and Rhode, 1958; Mullaney and Murphy, 1962), pigs (Szenti - Ivanyi, 1960; Zuffa and Skoda, 1960; McFerran and Dow, 1962; Csontos, 1964) and rabbits (Kaplan, 1959; McFerran and Dow, 1962; Pette, 1965). Also susceptible are cultures of testicular cells from calves (Csontos, 1964; Borgen and Bendixen, 1965), pigs (Kubin, 1969), human amnion (Gois and Mayer, 1959) and HeLa cells (Scherer and Syverton, 1954; Mullaney and Murphy, 1962; Sacco and Maglione, 1968), mouse embryos (Scherer, 1953) and fowl embryos (Ivanovics, Beladi and Szollosy, 1955, 1956; Zuffa and Skoda, 1960; Beladi, 1962; Rusev and Mateva, 1962). In addition to primary, secondary and certain established cell cultures, the virus of Aujeszky's disease grows readily in suspended cell cultures of the Maitland type. These include mouse embryo cells (Ivanovics et al, 1955), excised chorioallantoic membranes (Abraham, Ivanovics and Koch, 1954), fragments of fowl embryo tissues (Cserey-Pechany, Beladi and Ivanovics, 1951; Ivanovics et al., 1954) and HeLa cells (Sacco and Maglione, 1968).

Cell culture methods are ideally suited to large

scale experimental studies and, compared with the traditional methods of animal inoculation, their use in Aujeszky's disease investigations is preferred on humanitarian grounds. Moreover, many cell culture systems are just as sensitive to virus infection as are biological methods. This was clearly shown by McFerran and Dow (1962) who found that primary cultures of rabbit and pig kidney cells were as susceptible as rabbit inoculation for the isolation of Aujeszky's disease virus from clinical material whereas Masic and Petrovic (1964) observed that pig kidney and fowl embryo fibroblast cultures were more sensitive than rabbits and mice. Also, Rondhuis and Haagsma (1966), who isolated the virus from dog tissues using primary mink and pig kidney cells, regarded mink cells as being the more sensitive since the cytopathic effects developed more rapidly and the virus titres were higher than in pig kidney cells.

In certain types of cell cultures a number of serial passages of the virus are necessary before microscopic evidence of viral replication is obtained. For instance, Sacco and Maglione (1968) observed that in monolayers and suspensions of HeLa cells, field strains of the virus became established after 2-4 serial transfers. In the later passages, the virus titres

were as high as 10^7 $TCID_{50}$ per ml. Likewise, Popescu (1965) and Kabelik and Korysch (1968) obtained virus titres of 10^8 and $10^{9.5}$ $TCID_{50}$ per ml. respectively, in pig kidney cells after a few initial serial passages. On the other hand, the differences in virus titres are slight when cell culture-adapted strains are serially propagated in cultures of other cell types. For example, Zuffa and Skoda (1960) and Masic and Petrovic (1964) noted that the titres of cell culture-adapted virus in pig kidney and fowl embryo cells, were similar. Likewise, there were no significant differences in the values reported for dog kidney cells by Torlone (1958), for rabbit and pig kidney cells by McFerran and Dow (1962) for primary pig kidney cells by Kabelik and Korysch (1966) and for fowl embryo cells by Ivanovics et al (1954), Zhelov and Khristov (1962) and Svobodova, Sabo and Blaskovic (1970).

The cytopathic effects of pseudorabies virus are readily recognised and are often spectacular and infected mammalian cell cultures generally show foci of founded cells with, in some cases, syncytia and aggregates of nuclei containing single large eosinophilic inclusions of Cowdry type A (Scherer, 1953; Scherer and Suverton, 1954; Kersting et al., 1958; Kaplan and Vatter, 1959). These features are less conspicuous on monolayer and suspended cell cultures of fowl embryos

(Zhelev and Khristov, 1962; Rusev and Mateva, 1962) and may even be absent (Cserey-Pechany et al., 1951; Ivanovics et al., 1956). In such cultures infected embryo cells lose their regular spindle shape and become spherical or stellate with long retracting, often beaded processes. Proof that the different types of cellular changes are virus-induced may be obtained by the usual methods of specific virus neutralisation (Mayer, Skoda and Zavada, 1962; Zuffa and Skoda, 1962) and immunofluorescence (Albrecht, Blaskovic, Jakubik and Lesso, 1963).

Consecutive long-term passages in monolayer cultures of mammalian cells has little effect on the pathogenicity of recently isolated virus (Kerekjarto and Rhode, 1957; Torlone, 1958; Sacco and Maglione, 1968). On the other hand, serial passages in fowl embryo cell cultures may result in attenuation (Ivanovics et al., 1955; Bran, Suhaci and Ursache,1963) or complete loss of virulence for some natural and experimental hosts (Skoda, 1962; Zuffa, 1966), while some non-pathogenic strains may retain their immunogenicity even after prolonged serial passages in fowl embryo cells. (Skoda et al., 1964b), Adaptation to fowl embryo cell cultures of wild strains of Aujeszky's disease virus may reduce their ability to form syncytia in mammalian cell cultures (Zuffa and

Crigelova, 1966) or, as was shown by Beladi and Bakay, (1963), may give rise to latent, persistent infections in calf kidney cells.

Refractory Hosts:

It would appear from the limited data that is available that there is no transmission of the virus to amphibians (Remlinger and Bailly, 1934), although it has been reported that the virus may persist, without replication, in the brains of intracerebrally inoculated tortoises for up to five weeks (Remlinger and Bailly, 1933h), and it is probable, in these circumstances, that the virus survives passively.

CLINICAL ASPECTS

Natural and experimentally induced Aujeszky's disease is a well-defined clinical entity in most mammalian species but in pigs and rats the occurrence of clinical symptoms is inversely related to the age of the affected animal (Hirt, 1935; Vianello, 1942; Shahan et al., 1947a; Nikitin, 1960; Akkermans, 1963; Olander, Saunders, Gustafson and Jones, 1966; Csontos, 1969). Thus, adolescent and adult pigs often undergo a mild and subclinical infection (Lukashev and Rotov, 1939; Kojnok, 1962; Shope, 1964).

The early clinical descriptions of Aujeszky's disease are praiseworthy both for their accuracy and wealth of detail (Aujeszky, 1902; Koves, 1935; Lourens, 1935; Panisset, 1935; Remlinger and Bailly, 1938). Symptoms such as altered demeanour, excessive salivation and loss of deglutition due to pharyngeal and laryngeal paralysis are common to rabies and Aujeszky's disease, but the short course of the clinical illness, the lack of aggressive signs and the intense pruritus of the skin are the main clinical guide lines that distinguish pseudorabies from rabies.

In its fully developed state, Aujeszky's disease in ruminants, carnivores and the laboratory rodents is often of an explosive nature. There is, invariably, profound central nervous involvement that is dominated

by restlessness, staggering gait, tonoclonic convulsions and paralysis leading to exhaustion, prostration and coma. Hyperaesthesia of the skin is an unique and pathognomonic symptom in the afore-mentioned animals but may be entirely absent in the disease in older pigs (Shope, 1931, 1932; Laszlo, 1938; Hutyra, Marek and Manninger, 1938; Glover, 1938; Andrev and Uzunov, 1940; Bianchi, 1942; Bendixen et al., 1965).

A review of the clinical features of Aujeszky's disease in the different natural hosts is not within the scope of this present work but, since part of the dissertation was directed towards the behaviour of the virus in certain laboratory hosts, the relevant clinical aspects of the experimental disease in these animals will be considered.

In rabbits, guinea pigs, mice, mink and ferrets, the onset and duration of the clinical illness and severity of the symptoms are greatly influenced by the route of administration of the virus (Shope, 1931; Remlinger and Bailly, 1933c, 1933d; Ercegovac, 1960; Goto et al., 1968) but the clinical features of the disease in the different species are mostly very similar except that rabbits probably react more severely than other animals.

In rabbits, the first clinical symptoms of the disease following subcutaneous inoculation of the virus

are uneasiness, anxiety, anorexia and disinclination to move. These are quickly followed by progressive nervous excitement, fright, grinding of teeth, hypersalivation and scratching or gnawing at the site of inoculation. The regional pruritis increases so rapidly that the symptoms of scratching and biting become persistent. The skin over the inoculated region is soon bereft of hair and the underlying tissue manifests hyperaemia, haemorrhage and oedema, "Pari passu", there is elevation of temperature, laboured stertorous breathing and muscular tremors. Soon the animal lies exhausted on its side, is unable to rise and death supervenes 6 to 29 hours after the onset of pruritus which may be preceded by loss of consciousness and clonic convulsions (Shope, 1931).

The onset and course of the illness following intracerebral injection is generally brief (Shope, 1931; Hurst, 1933; Remlinger and Bailly, 1933d). Symptoms of nervous excitement are pronounced and most affected animals run wildly to and fro and often butt their heads against the cage doors. In Shope's studies some rabbits also developed bilateral blindness although there was neither hyperthermia nor pruritus. In most animals the induction of respiratory failure and coma was rapid.

Symptoms following intraocular inoculation are similar to those that develop after intracerebral or subdural infection (Remlinger and Bailly, 1933d).

When the virus is instilled into the nasal cavities there is usually a significant delay in the onset of illness and symptoms of nervous excitement are less prominent. Fever is absent and rabbits often die suddenly without showing symptoms of the illness (Shope, 1931).

In the experimental disease in rabbits the development of pruritus in relation to the route of infection is discussed by Hurst (1933) who defines pruritus as "a referred sensation arising from a central lesion", the lesion in most cases being located in the corresponding spinal ganglion.

In their comparative study of the responses of adult rabbits, mink and ferrets to subcutaneous infection with two virulent strains of pseudorabies virus Goto et al (1968) noted that the clinical features of the disease were severe and similar in the first two species. Ferrets showed relatively milder symptoms and ninety-two per cent died either without showing obvious symptoms or on the day following the onset of illness. The course of the infection was more rapid in rabbits and mink than in ferrets, but itching at the inoculation

site and bleeding from the mouth due to tongue biting occurred in all three species. On the other hand, nervous symptoms such as opisthotonus, spasms, extension of the legs and papillary changes were present only in mink and rabbits. In all three species, the length of incubation period and the time of death were inversely related to the dose of virus inoculated but the duration of clinical disease showed no correlation with the amount of the inoculum. No marked differences were noted in the clinical signs or the course of infection in animals inoculated with the two strains of virus (Goto et al, 1968).

In experimental studies of the responses of rats to the virus by Schmiedhoffer (1910), Shope (1931), Remlinger and Bailly (1933c), Nikitin (1960) and McFerran and Dow (1970) differences in the onset, nature and severity of clinical signs were related to the routes of inoculation of the virus. For instance, salivation and anorexia were constantly observed in the orally infected rats but these symptoms did not appear following subcutaneous infection (Schmiedhoffer, 1910). However, pruritus was a common feature in rats infected by either route and in Nikitin's studies 63 per cent of the intramuscularly infected rats showed pruritic lesions.

Similarly, guinea pigs (Shope, 1933; Reagan et al,

1957) and adult hamsters (Reagan et al, 1952) inoculated by different peripheral routes showed the characteristic symptoms of scratching and biting at the inoculation sites, excessive salivation, dyspnoea and prostration.

Most grey and white mice inoculated intraperitoneally (Schmiedhoffer, 1910; Isobolinski and Patzewitsch, 1912; Shope, 1931;) and white mice injected subcutaneously (Traub, 1933) showed nervous and respiratory symptoms but in Traub's studies some mice that did not show pruritus, developed predominantly respiratory symptoms and died. Similarly, 1 to 3 days' old rabbits showed respiratory symptoms in 24 - 72 hours when inoculated subcutaneously with cell cultured virus (Maglione and Sacco, 1969). On the other hand, unweaned hamsters injected by different routes showed predominantly nervous symptoms 3 - 6 days later (Reagan et al, 1957).

Dempsher, Larrabee, Bang and Bodian (1955) and Dempsher, Tokumaru and Zabara (1959) discussed the physiological and pharmacological aspects of itching in experimentally infected rats and noted that scratching of the eye occurred about 48 hours after injection of the virus into the vitreous humour. Histological changes were found in the ipsilateral superior cervical ganglion cells from which the virus was recovered. The affected

cells discharged intermittent groups of impulses firstly over the preganglionic nerve and later simultaneously over the postganglionic nerve fibres. In time, the postganglionic nerve fibres became silent, the sympathetic transmission failed, but the impulses continued to be discharged prominently over the preganglionic nerve fibres. In view of these results, it was postulated that pseudorabies virus infection may possibly interfere with the functioning of an inhibitory system originating in the central nervous system and which exerts its effects on the presynaptic nerve ending. It was also suggested that some of the chemical constituents of this system were adrenaline, non-adrenaline and gamma-aminobutyric acid (Dempsher et al, 1959).

The propensity to invade the nerve cells and engender sensory lesions in some of the mammalian hosts is perhaps a genetic trait of the virus. Serial passages in heterologous hosts such as fowl embryos (Glover, 1939; Burnet et al, 1939; Skoda, 1962) and pigeons (Toneva, 1961) entail a reduction or loss of this property (Bognar and Kucsera, 1965). The occurrence of the so-called "low-pathogenicity" variants in the respiratory tract of the pig, a host that does not generally develop pruritic lesions in response to inoculation with the virus, might support this assumption

The presence or absence of pruritic responses in the hosts might also reflect differences in the innate efficiency of the inhibitory mechanisms postulated by Dempsher et al (1959). The development of significantly high levels of histamine in the venous blood of experimentally infected rabbits soon after the onset of pruritus as reported by Becker and Rahn (1965) must be regarded as a closely related biochemical lesion.

Clinical descriptions of the experimental disease in fowls, pigeons and other avian species are meagre. Most studies emphasize the development of nervous symptoms, such as excitement, muscular tremors, torticollis, paralysis of the muscles of the wings and thighs and stupor, but neither pruritus nor alterations of body temperature have been reported (Nicolau et al, 1937a; Shahan et al, 1947b).

PATHOGENESIS

NATURAL INFECTIONS

The pathogenesis of the natural disease is not fully understood and very little of the published data correlates virus recovery with the course of the clinico-pathological events. However, the results of some experimental studies do enable us to speculate on the possible sequence of events in naturally infected animals.

In 1966, Csontos and Szecky observed that 15 to 20 per cent of pigs that succumbed to the natural disease had naso-pharyngeal lesions and that the incidence of tonsillary lesions was three times greater. They also noted that virus induced changes in the pharyngeal mucosa were distributed primarily in the lymph follicles. Abnormalities in the squamous stratified epithelial cells in the buccal part of the pharynx included "megalocytosis", "karyomegalia" and acidiphilic nuclear inclusions. Examination of the palatine tonsil showed that the inflammatory and necrotic changes were more pronounced in the lymphoid follicles while the tonsillar fossae showed degenerative changes in the epithelial cells as well as syncytia and intranuclear inclusions. The tonsillary epithelium appeared to be affected later (Csontos and Szecky, 1966). The presumption that viral replication first occurred in the

lymphoid tissues of the naso-pharynx and the tonsils was supported by virological data, and virus titres in the nasal and pharyngeal mucosae and lungs were significantly lower than the titres in the so-called Waldeyer's tonsillar ring (Csontos, 1966). In general, the virological findings were in harmony with those of Masic, Ercegan and Petrovic (1965a) who postulated a haematogenous spread of the virus following primary replication in the tonsillary tissue.

Experimental Infections:

General: Pseudorabies virus behaves differently in different species of animals and Tokumaru (1957), Bodon et al (1968), Tatarov (1968) and Tatatrov and Cergov (1969), demonstrated the existence of naturally occurring strains that differed markedly in their tissue affinities. Moreover, the results of electrophoretic mobility studies of strains of pseudorabies virus (Matis and Zuffa, 1969) have shown that individual strains are composed of an non-homogeneous population of virus particles with possible differences in their host affinities. In the pig (Csontos and Derzsy, 1944; Becker, 1961; Olander et al, 1966), the rabbit (Hurst, 1933, 1934; Becker, 1966, 1968; Maglione and Sacco, 1969) and the rat (Nikitin, 1960; McFerran and Dow, 1970), and also in some species of wild mammals (Trainer

and Karstad, 1963), the virus exhibits pleuricellular affinities. On the other hand, in cattle and sheep, and possibly in domestic carnivores, the virus appears to be strictly neurotropic (Dow and McFerran, 1963; McFerran and Dow, 1964a, 1964b; Olander et al, 1966). The behaviour of the virus in monkeys is said to be similar to that in cattle (Hurst, 1934), but little is known about the pathogenesis of the disease in small rodents and birds.

Rabbit: Hurst's studies (Hurst, 1933, 1934) on the pathogenesis of pseudorabies in the rabbit are amongst the most significant contributions to our present knowledge of the host-pathogen interactions in the herpesvirus group. Pseudorabies virus is pantropic in rabbits and, after intramuscular, intradermal and subcutaneous inoculations, it reaches the central nervous system along the peripheral nerves. Virological and histological studies suggest that there is an ascending spread of infection within the central nervous system and that the virus diffuses centrifugally to the lower segments of the cord following intracerebral inoculation. Replication of the virus in the Schwann cells of the peripheral nerves, the neurons and satellite cells of the spinal ganglia have been demonstrated in the electron microscopic and by the autoradiographic studies of

Becker (1968) and Zipper and Becker (1970), respectively. Moreover, the hypothesis of a centripetal neural transmission of the virus from the peripheral sites to the cord has been confirmed by the studies of Hirt (1942) and Bergman and Becker (1969).

In experimentally infected rabbits, the virus of Aujeszky's disease can also be recovered from the circulating blood and from the spleen, lungs, lymph nodes, kidneys and adrenal glands, but the presence of viraemia may depend on the strain of virus used (Hurst, 1933, 1934). When massive doses of virus are inoculated intravenously the resulting viraemia lasts for about two hours whereupon the virus is rapidly filtered into different viscera resulting in the establishment of multiple infective foci. From these foci the virus travels along the peripheral nerves to reach the spinal ganglia. The ability of the virus to invade the blood was elegantly demonstrated by inoculating the virus subcutaneously into an area of skin deprived of its nerve supply. Following a short delay in the onset of pruritic symptoms, the virus appeared simultaneously in the blood, lungs, spleen and the spinal cord (Hurst, 1934).

Aujeszky (1902), Schmiedhoffer (1910), Zwick and Zeller (1911), Isobolinsky and Patzewitsch (1912), Sangiorgi (1914), Bang (1932) and Manninger and Marcis (1937) were impressed by the high concentration of virus

found in the blood of infected rabbits which suggested that haematogenous spread was a possible method by which the virus reached the central nervous system. Unfortunately, this assumption was not supported by experimental evidence. Viraemia has also been frequently demonstrated in experimentally infected guinea pigs (Lamont, 1947), mice (Masic et al, 1965b) and fowls (Remlinger and Bailly, 1933e; Jonnesco, 1934), but is seldom found in pigs (Toneva, 1958; Masic, 1961; Jamrichova and Skoda, 1969), sheep (Toneva, 1958), cattle (Braga and Faria, 1932), cats and dogs (Remlinger and Bailly, 1938). In rabbits and mice the virus may become attached to the erythrocytes (Remlinger and Bailly, 1933c; Reagan et al, 1954) but this observation has yet to be confirmed. McFerran and Dow (1965) isolated Aujeszky's disease virus from 2 of 500 samples of pig blood and suggest that the viraemic phase in the pig probably reflects the amount of virus in the circulating leucocytes and that, as in bovine rhinotracheitis infection (McKercher, Saito, Wada and Straub, 1958) the leucocytes probably carry the virus from the initial foci of replication to other predilection sites.

Pig: The pathogenesis of experimentally induced pseudorabies in the pig has been studied in detail by the Northern Ireland and Czechoslovakian groups of workers. In piglets infected orally (Sabo, Rajcani

and Blaskovic, 1968b) or intranasally (McFerran and Dow, 1964; 1965; Sabo et al, 1969), there is unequivocal evidence of primary viral replication in the epithelium of the nasopharyngeal and tonsillar mucosae. Sabo et al (1969) studied the pattern of virus distribution by infectivity assays and immunofluorescence and reported high titres of virus, reaching 10^8 $TCID_{50}$ per gram of tissue, as well as nuclear and cytoplasmic localisation of the viral antigens and inflammatory changes in the naso-pharyngeal and tonsillar mucosae at the 24th and 48th hours respectively. Entry of the virus into the brain probably occurs within 48 to 72 hours post infection. According to McFerran and Dow (1965) and Sabo et al (1969) the virus may reach the brain by either or both of the following routes viz. from the nasal mucosa along the fila olfactoria to the olfactory lobes or from the pharynx along the glasso-pharyngeal nerves to the nucleus solitarus in the medulla. The existence of a third pathway along the trigeminal nerve to the medulla and pons is also possible (McFerran and Dow, 1965; Sabo et al, 1968b, 1969).

Similar mechanisms of spread have been reported in experimental pseudorabies of rats (Sabo and Sadecky cited by Sabo et al, 1968b) and mice (Sabin, 1938)

except that the olfactory pathway may not be involved in mice.

Whereas the evidence for haematogenous spread in the pig is only slight (Sabo et al, 1968b), immuno-fluorescent studies indicate that virus replication takes place in the lymphoid tissue of the pharynx and tonsillar crypts (Sabo et al, 1968b, 1969). Also from the second day onwards, virus titres increase in the cervical lymph nodes and moderate to high titres occur in the remote lymph nodes by the 4th or 5th day due, perhaps, to spread of infection via the lymphatics. Similar findings have been reported earlier by Taga, Berbinschi, Cirstet and Coman (1956, 1957).

Histological evidence of virus activation suggests that there is a rapid centrifugal spread of the virus within the central nervous system (McFerran and Dow, 1965; Sabo et al, 1968b, 1969). In intransally infected piglets, the neurons and glial cells of the olfactory bulbs display diffuse immuno-fluorescence from the second day onwards (Sabo et al, 1969) but other parts of the cerebral cortex do not show virus activity at this time. In these animals, the virus titres in the 5th cranial nerve and the olfactory bulbs are not only very similar but increase steadily from the second day onwards, while in perorally infected animals, the titres in the olfactory bulbs develop on

the 4th day. Evidence of viral replication in the cerebral cortex appears only on the 4th or 5th day (Sabo et al, 1968b).

Virus can also be recovered from the lungs 24 hours post infection and from the spleen, kidneys and adrenal glands shortly afterwards (Sabo et al, 1968b, 1969; McFerran and Dow, 1965). The presence of virus in the lungs has been largely attributed to direct extension of infection from the upper respiratory tract (Sabo et al, 1969) but the importance of macrophages in the transport of the virus from the nasopharyngeal mucosa to the other viscera has also been stressed. Also, McFerran and Dow (1965) hinted at a possible neural spread of the virus from the lumbo-sacral cord to the adrenals but they did not recover the virus from the urine or faeces.

Experimentally infected pigs excrete the virus in the oral and nasal secretions for considerable periods (McFerran and Dow, 1965; Sabo, 1969; Sabo and Blaskovic, 1970). Moreover the amount of virus present in the tonsillar tissue and in the cervical lymph nodes declines linearly with time and the disappearance of the virus coincides with the appearance of neutralising antibodies in the circulation (Sabo and Blaskovic, 1970).

Sabo (1969) inoculated adolescent pigs subcutaneously with two doses of the live, 'BUK' vaccine, and

challenged them perorally with a high dose (10^6PFU) of the virulent virus on the 30th day when the animals had a median neutralising antibody titre of 1:12. Virus was recovered from the naso-pharynx of most pigs, up to the 9th day post inoculation and forty per cent excreted the virus until the 13th day. All pigs excreting virus had a significant anamnestic increase in the neutralising antibody titres, and, in one animal, excretion of the virus continued up to the 20th day with a concomitant steady increase in the antibody titre. Other evidence of the co-existence of virus and neutralising antibodies in pigs was reported by Sabo and Blaskovic (1970).

The distribution of pseudorabies virus in piglets infected subcutaneously differs considerably from that in piglets infected orally or intransally. Both virulent and attenuated strains of the virus replicate at the sites of injection (Popovici, Taga, Berbinschi, Cirstet and Coman, 1956; Jamrichova and Skoda, 1969; Rajcani, Sabo and Blaskovic, 1969) and both types invade the central nervous system but, from the results of the studies of Jamrichova and Skoda (1969) in adolescent pigs, it would seem that the vaccine virus does not multiply in the drainage lymph nodes and that it is passively transported there from the subcutaneous

tissue by way of the lymphatics. Nor is there any evidence that vaccine virus replicates in the internal organs.

Results of immunofluorescent studies reveal that, at the injected site, the virulent virus penetrates into the fibrocytes, adipose tissue cells, macrophages, muscle cells, endothelial cells of venules and the endoneural cells of the small nerve fibres. Specific fluorescent staining has also been detected in the Schwann cells and endoneural fibroblasts of nerve bundles. The fact that the axoplasm is unaffected, suggests possible lymphatic spread of the virus in the nerve bundles. There is also evidence that the virus can be transported to the spinal cord along the ventral and dorsal nerve routes and that replication of the virus may take place in the glial cells of the anterior, lateral and posterior columns and, later, in the glial cells and neurons in the gray matter.

Ox and Sheep: A limited number of investigations have been carried out in ruminants with the object of correlating data of virus distribution with the anatomical and histological findings, but quantitative virological data are lacking. The results of these studies suggest that the virus of Aujeszky's disease in cattle and sheep is strictly neurotropic (Dow and McFerran, 1964, 1966; McFerran and Dow, 1964a, 1964b).

In cattle, the lesions are indicative of an ascending spread of the virus along the peripheral nerves to the related ganglia and thence to the spinal cord or brain, depending on the site of inoculation. Virus has not been recovered from the blood, cerebrospinal fluid or any extraneural sites. Following oral infection, virus can be recovered from the pharynx but its presence in the oral secretions is debatable (Shope, 1931; Tepper, 1960). Moreover, McFerran and Dow (1964a) have postulated that the virus is attached to the nerve elements in the pharyngeal mucosa.

Following cutaneous inoculation, the distribution of pseudorabies lesions in the central nervous system of sheep is similar to that in cattle and is consistent with a centripetal spread of virus along the peripheral nerves to the dorsal root ganglia (Dow and McFerran, 1964). This pattern of neural spread is well illustrated by the intraconjunctival, oral and nasal routes of infection (Dow and McFerran, 1964).

In sheep, the pattern of virus distribution also appears to be related to the dose of the inoculated virus. Thus with higher doses of virus the typical "mad-itch" syndrome develops and the distribution of the virus and the lesions would seem to suggest dissemination of virus along the cutaneous nerves to the

dorsal root ganglia (Dow and McFerran, 1966). Virus distribution in the viscera is minimal. However, with the lower doses of the virus, the correlation between the severity of the lesions and the distribution of the virus within the nervous system is less evident and, in some instances, dissemination of the virus may occur in the internal organs, similar to that observed in calves infected intravenously (Dow and McFerran, 1966).

Cat and Dog: There are very few accounts of the experimental disease in dogs, cats and other carnivores. However, Sabo et al (1968a) observed that the spread of pseudorabies virus in perorally infected cats is similar to that in perorally infected pigs. He also noted that primary virus replication occurs in the pavement epithelium of tonsils and the pharyngeal mucosa.

CYTOPATHOGENESIS

Virological Aspects: Much of the present information on the dynamics of the growth cycle of pseudorabies virus in susceptible cells stems from the pioneering investigations of Kaplan and his colleagues. Studies on monolayers of a continuous line of rabbit kidney cells (Strain RK 13) showed that 50 per cent of the input virus is absorbed in 30 minutes at 37°C (Kaplan and Vatter, 1959) while absorption in fowl embryo cells is

nearly complete in 2 hours (Beladi, 1962; Albrecht et al, 1963; Zuffa, Matis and Salaj, 1970). Absorption is followed by penetration of the virus into the cell and there is indirect evidence that the mechanisms of penetration are energy-mediated. The process fails to take place in the presence of graded amounts of cyanide (Kaplan, 1962). However, information is lacking regarding other parameters of absorption and penetration such as the influence of temperature, electrolytes, inoculum size and the relative sensitivities of the different cell types.

The duration of the "eclipse phase" is approximately 3 hours (Kaplan and Vatter, 1959) and the latent period ranges from 4-5 hours in monolayer and suspension cultures of RK 13 cells (Kaplan and Vatter, 1959). On the other hand, in fowl embryo monolayer and suspension cultures the values range from 2 to 4 hours and 6 to 8 hours respectively (Mayer, Skoda and Zavada, 1962). Using a high multiplicity of infection Albrecht et al (1963) observed that the latent period of infection of fowl embryo monolayer cultures was 2 hours and 40 minutes. When single rabbit kidney cells were infected and then incubated in isolated drops of nutrient medium the latent period ranged from 6 to 10 hours and this variation was thought to be due to an

asynchrony in the initiation of virus synthesis in individual cells or in maturation of the virus in different single cells (Reissig and Kaplan, 1960). However, the reasons for the variations in the time course of the latent period in singly infected individual cells are not fully understood.

Data are lacking on the time of release of the mature virus particles from infected cells. The kinetics of virus synthesis appears to be exponential although the underlying mechanism is not clear (Kaplan, 1966). While DNA is the only component in the cells known to duplicate exponentially, the studies of Kaplan (1962) with herpes simplex indicate that DNA is not responsible for the logarithmic increase of mature virus and is probably not the limiting factor in the maturation process. An exponential synthesis of some enzymes or precursors necessary for virus maturation has been postulated (Kaplan, 1966) but this suggestion requires to be confirmed before it can be said to apply in pseudorabies infections.

With high absorbed multiplicities of infection peak virus titres are obtained in 12 hours in RK 13 cells (Kaplan and Vatter, 1959) and in 14 to 16 hours in fowl embryo cells (Wawrzkiewicz, 1969) but, with low multiplicities of infection in fowl embryo cells, the

rise in virus titres begins at 12 hours and maximum yields are obtained at 48 hours (Albrecht et al, 1963). Suspensions of rabbit kidney cells yield approximately 1,000 plaque forming units (PFU) per cell (Kaplan and Vatter, 1959), whereas the yield from individual cells varies from 63 to 500 PFU per cell (Reissig and Kaplan, 1960). The differences in the yield rates have been attributed to differences in the cultural conditions of single cells as compared with cells in suspension.

Morhpological Aspects: The nature of the structural changes in pseudorabies-infected cells in the post-absorptive phase is not known, but it is possible that the cellular events are similar to those demonstrated in herpes simplex infection of baby hamster kidney cells, (Strain BHK 21) by Holmes and Watson, (1963). Enveloped virions of herpes simplex are absorbed and penetrate into the cells more readily than naked particles, and there is recent evidence that this is also true of pseudorabies virus in BHK 21/13 cells (Darlington and Moss, 1969). The absorbed particles are ingested into pynocytotic cytoplasmic vesicles whence they appear to migrate towards the nucleus. In pseudorabies the precise sequelae to the absorption of the virus particles are not known but it seems likely, from the studies of Schulze and Benndorf (1963) and

Felluga (1964), that the progeny viral particles are synthesised in the nucleus in the same way as herpes simplex particles are.

In general, therefore, the development pattern and morphological characteristics of the different viral forms and the nuclear and the cytoplasmic structural alterations are reminiscent of those observed in other herpetic infections (Morgan, Ellison, Rose and Moore, 1954; Reissig and Melnick, 1955; Luse and Smith, 1958; Bracken and Norris, 1958; Watrach, 1962).

Immuno-cytological Aspects: One of the earliest accounts of the use of immunofluorescence for the detection of specific viral antigens in pseudorabies infected cells is that of Albrecht et al (1963) who sought to correlate the stages of development of the viral antigens with the morphological and tinctorial changes in the infected cells, as seen by light microscopy. Fowl embryo monolayer cultures infected with a high dose of the BUK strain of the virus produce small particles of specific fluorescence in the nuclear matrix within 5 hours. After 8 hours, the nuclear fluorescence appears distinctly multifocal and granular although larger spherical fluorescent bodies may also be seen. Large areas of the monolayer may be affected, and in some of the infected cells the entire nuclear matrix is filled with fluorescent material. The

morphological changes in the infected cells up to this stage consist of a progressive swelling of the nucleoli which become eccentric and finally disappear. Margination of disrupted chromatin is evident at 8 hours post inoculation but there is no evidence of structures resembling intranuclear inclusions. After 12 hours the distribution of the viral antigens is manifest as granular fluorescent masses in the perinuclear zone of the cytoplasm or as fluorescent aggregates in the region of the Golgi apparatus; pari passu there is a reduction in nuclear fluorescence. At this time, cell transformation takes place and the fibroblasts lose their characteristic spindle form and become stellate or spherical in shape. Cellular transformation is associated with nuclear pyknosis and an overall reduction in the size of the cell, these features being especially prominent at the 24th hour post infection. Between the 16th and 24th hours, various degenerative changes occur, including vacuolation of the nucleus and cytoplasm. According to Albrecht et al (1963) the logarithmic phase of virus development coincides with the stage of bright nuclear fluorescence whereas the appearance of cytoplasmic fluorescence is not accompanied by a concomitant rise of the total virus titres. Thus, they surmise that the bulk of the

fluorescent material in the cytoplasm probably represents non-infectious virus protein.

The immuno-cytological findings of Albrecht et al (1963) have been confirmed by Strel'tsov, Scherbatykh and Noskov (1967) and Wawrzkiewicz (1969). The latter also studied the effects of sodium salicylate and morfolinobiguanid on the replication of the virus in primary calf kidney cells, since these substances are known to inhibit virus replication. The results showed that nuclear and cytoplasmic fluorescence were weak and that the time taken for their development was significantly delayed, which suggests that suppression of viral antigen synthesis had taken place. The findings are in agreement with their autoradiographic data and the marked suppression of virus titres obtained in the treated cultures.

Chemical Aspects: Information about the cyto-chemical aspects of the mode of synthesis of pseudorabies virus is fragmentary but the available data suggest that the mechanisms of virus synthesis are similar to those of bacteriophages (Kaplan, 1966).

Pseudorabies-infected cells synthesise viral DNA far in excess of the amounts required for the formation of mature virions and only about 20 per cent of the synthesised viral DNA is integrated with the virus

particles (Ben-Porat and Kaplan, 1963). Furthermore, as in bacteriophage systems, there is a precursor pool of viral DNA from which DNA is withdrawn at random and integrated into the virion (Ben-Porat and Kaplan, 1963). There is also clear evidence that the viral DNA replicates semi-conservatively (Kaplan and Ben-Porat, 1964).

Infection of RK 13 cells with pseudorabies virus produces an increase in the activity of the enzymes involved in the synthesis of viral DNA, and it is especially interesting to note that one of these enzymes, thymidine kinase, is antigenically different from that found in uninfected cells (Nohara and Kaplan, 1963; Hamada, Kamiya and Kaplan, 1966). It seems likely, therefore, that such enzymes might constitute part of the virus-specific antigens.

Shimono and Kaplan (1968) and Stevens, Kado-Boll and Haven (1969) related the inhibition of host-cell DNA in RK 13 cells infected with herpes simplex or pseudorabies virus to suppression of histone synthesis. They also showed that five major virus-induced acid-extractable proteins appear in the nuclei of pseudorabies infected cells four hours after infection. Four of these are virus structural proteins while the fifth is probably host-derived protein. Stevens et al (1969) postulated

that these proteins have a positive role in the mechanism of inhibition of host cell DNA in virus-infected cells.

Sydiskis (1970) determined the intracellular localisation of pseudorabies virus particles and sub-virion components at various stages in the growth-cycle. At different intervals after infection nuclear and cytoplasmic extracts were prepared from the infected pig kidney monolayers using two different radioisotopes. Their results showed that, at 6 hours, 85 per cent of the total infectious virus was associated with the nuclear extract, whereas at 12 hours the value had dropped to 26 per cent. Concurrently, the amount of infectious virus in the cytoplasmic extract increased from 15 per cent at 6 hours to 74 per cent at 12 hours post infection.

The sub-virion components were associated with the nuclear extract for at least 10 to 24 hours after the infection and the growth-cycle ended at 16 hours. The difference in the densities noted between the nuclear and cytoplasmic virions indicated that the virus found in the nucleus was not fully enveloped, and is reminiscent of the findings reported for herpes simplex infection by Spring and Roizman (1968).

PATHOLOGY

In general there are no pathognomonic anatomical alterations at necropsy.

The histopathology of the natural and experimental disease in the pig is well-documented and has similar features. The chief lesion is a diffuse, lympho-histiocytic, non-suppurative, meningo-encephalomyelitis and ganglioneuritis characterised by moderate to pronounced neuronal and glial necrosis, diffuse or focal gliosis and marked perivascular cuffing (Salyi, 1935; Hirt, 1936; Dow and McFerran, 1962a; Mackay et al, 1962; Knosel, 1965; Olander et al, 1966). The cerebrum is invariably affected severely, the cellular exudation being most prominent in the corpus medullare around the lateral ventricles. The lesions diminish in intensity caudally (McFerran and Dow, 1962 ; Olander et al, 1966) and intranuclear herpetic inclusions are usually recognised (Link, 1933; Gordon and Luke, 1955; Done and Venn, 1958; Nikolitsch, 1958; Dow and McFerran, 1962a).

Unlike the disease picture in pigs, the neuropathology of natural and experimentally induced pseudorabies in ruminants and carnivores is highlighted by a predominant involvement of the lumbo-sacral segments of the cord and the related dorsal root ganglia on the affected side (Dow and McFerran, 1962a, 1962b, 1963; Olander et al, 1966; Bergmann, 1968). Neuronal damage

is generally extensive and Cowdry type A nuclear inclusions are prominent. Lesions of meningo-encephalomyelitis are progressively milder or even trivial in the cervical segments of the cord and in the brain stem.

Among the experimental laboratory animals the tissue response to Aujeszky's disease virus is most spectacular in the rabbit. There is a fulminant local inflammation and necrosis at the injected site in which the nerve fibres in the fascia and musculature are severely involved (Hurst, 1933; Nicolau et al, 1937b; Lepine and Sauter, 1937; Olander et al, 1966). There is unequivocal histological evidence of spread of infection along the peripheral nerves interstitially and/or by the axis-cylinders to the corresponding spinal ganglia and the segments of the spinal cord where necrosis of the nerve cells and glia cells occurs in the dorsal horns. Intranuclear inclusions are detected in cells derived from all embryonic layers, namely neurons, glia cells, vascular endothelial cells, Schwann cells and sarcolemmal cells (Hurst, 1933).

The lesions in experimentally infected guinea pigs and mice are generally milder than those in rabbits (Hurst, 1933; Nicolau et al, 1937b; Shahan et al, 1947b). In rats there is no regular pattern of distribution of the lesions in the central nervous system consistent with

the route of infection (McFerran and Dow, 1970). Published accounts of the pathology of the disease in birds are meagre (Nicolau et al, 1937a, 1937b; Shahan et al, 1947b).

CYTOPATHOLOGY

Traub (1933) who first cultivated the virus in cultures of minced rabbit and guinea pig testicular tissue noted that the cytopathological features of pseudorabies infection were similar to those of Virus III in rabbits (Andrews, 1929a, 1929b) and herpes simplex infections in man (Andrews, 1930). Since then Aujeszky's disease virus has been propagated in cell cultures from diverse animal species and fowl embryos. Although the literature on the subject has been reviewed in detail by Lautie (1969), detailed descriptions of the cytopathology of the infection are lacking.

Mammalian Cell Cultures

In most culture systems, high multiplicities of infection of the virus generally induce characteristic cytopathic changes (CPE) in 24 to 36 hours (Kabelik and Korych, 1966; Bodin and Greczi, 1966) and there seems to be general agreement that the onset and progress of the cellular changes are faster in rabbit and pig kidney cells than in other mammalian cell systems (Lautie, 1969). For instance, Scherer and Syverton (1954) and Cserey-Pechany et al (1962) observed that the cellular lesions were not generally recognisable until the 3rd day, or later, in infected HeLa and monkey kidney cells. However, it is possible that the reported differences in the sensitivities of the various cell systems are more

apparent than real, because serial passages of field isolates often result in a marked reduction in the time of onset of the CPE, irrespective of the type of cell culture used (Kerekjarto and Rhode, 1957; Sacco and Maglione, 1968). Moreover, in some studies, the onset of the CPE appeared to be directly related to the dose of the virus (Kerekjarto and Hieronymi, 1958; Kabelik and Korych, 1966).

There is also a dearth of information regarding the influence of the composition of the culture medium and the temperature of incubation on the rate of growth of the virus and the nature of the CPE in mammalian cell cultures. Scherer (1953) who studied pseudorabies virus infection in a 'pure line' of L cells, reported that the virus titres were significantly higher when the cells were maintained in a calf serum ultrafiltrate medium than in horse serum-embryonic extract medium although there were no apparent morphological differences in the CPE. He also reported that virus multiplication was poor when the cultures were held at 22 to 25°C and ascribed this to the reduced metabolic activities of the cells.

Growth of pseudorabies virus in mammalian cell cultures is manifested by two distinct types of cytopathic change, the rounding and ballooning of infected

cells and the formation of syncytia (Scherer, 1953; Scherer and Syverton, 1954; Mullaney and Murphy, 1962). In demonstrating the two types of cytopathogenicity in monkey kidney cells infected with an avianised strain of virus, Tokumaru (1957) postulated that the original stock of virus contained two different strains of virus particle, the so-called 'G' and 'L' strains. The strains were separated by selective passage at limiting dilutions and whereas the 'G' strain produced the rounding and ballooning effect the 'L' strain caused the cytolytic giant cell formation. There were no apparent differences between the two strains either in the pattern of growth, heat stabilities or in their ability to induce intranuclear inclusions but they did differ in their neuropathogenicity for rats and in the size of plaques induced in monkey kidney cells. The 'L' strain produced large plaques and caused pruritic lesions in rats whereas the 'G' strain was characterized by the production of small plaques, and a greatly reduced neurotropic affinity.

The cytological and cytochemical aspects of the growth of pseudorabies virus were studied in detail by Kaplan and co-workers (Kaplan and Vatter, 1959; Kaplan and Ben-Porat, 1959, 1960, 1963; Reissig and Kaplan, 1960). In rabbit kidney monolayers infected with an

absorbed multiplicity of 5, or greater, of the virus, a few foci of necrosis appeared in less than 5 hours. The foci consisted of rounded cells with pyknotic and hyperchromatic nuclei and granular cytoplasm. This was soon followed by syncytial formation (Kaplan and Vatter, 1959) and proliferation of the nuclei, resulting in a marked progressive increase in the number of binucleated and trinucleated cells in the affected monolayers. The syncytia were thought to be due to the coalescence of cell nuclei rather than fusion of the cytoplasm of contiguous cells, although the process was preceded by lysis of the cell membranes. The increase in cellular DNA synthesis that occurred after the infection seemed to be related to the phenomenon of nuclear proliferation (Kaplan and Ben-Porat, 1959). These observations were confirmed and extended by Reissig and Kaplan (1960) in their studies in the kinetics of the replication of pseudorabies virus in single rabbit kidney cells.

In isolated rabbit kidney cells, nuclear division was first noted 7 hours after the infection and cells containing several nuclei were joined by thread-like extensions of the nuclear membrane. There were no mitotic figures, however, nor was there division of the cells and the effect was reminiscent of the amitotic division observed in herpes simplex (Gray, Tokumaru,

Scott and McNair, 1958) and Varicella infections (Weller, Witton and Bell, 1958). The nuclear division and the rate of DNA synthesis were significantly higher in single cells derived from older rather than younger monolayer cultures. The increase in DNA synthesis was elegantly demonstrated by the increased uptake of C^{14}-labelled thymidine by Reissig and Kaplan (1960) who showed, nevertheless, that cells obtained from both older and younger types of cultures supported virus growth and gave rise to syncytia and intranuclear inclusions. They also postulated that amitosis of virus infected cells was the result of increased synthesis of DNA coupled with suppression of mitosis.

In a more detailed study of pseudorabies infection in RK 13 cells, Kaplan and Ben-Porat (1963) separated viral DNA from host cell DNA by centrifugation in Cesium chloride density gradients and showed that, in the exponential phase of virus replication, there was a progressive decrease in the rate of incorporation of C^{14}-labelled thymidine into cellular DNA with a concomitant increased uptake by the viral DNA. The decrease in the rate of incorporation of the thymidine-2-C^{14} into cellular DNA was attributed to the inhibition of the synthesis of cellular DNA in virus-infected cells. Kaplan and Ben-Porat (1963) suggested that the migration

of the cellular DNA towards the periphery of the nucleus, which occurred in the first three hours of infection might be responsible for the inhibition of cellular DNA synthesis and gave autoradiographic evidence in support of this assumption. In the development of Cowdry 'A' type intranuclear inclusion bodies, margination of the nuclear chromatin was a principal feature but, since the shift in the grains of the cellular DNA towards the periphery of the nucleus occurred before there was any visible formation of inclusion bodies, the two phenomena were considered to be unrelated (Kaplan and Ben-Porat, 1963).

The possibility that the type of cytopathic effect is related to virulence has been investigated by a number of workers. For instance, Bodon et al (1968) observed that the growth of virulent and 'low-pathogenicity' strains of virus recovered from swine pneumonia cases was characterized by two types of CPE as shown by acridine-orange fluorescence. The virulent strains induced the formation of large syncytia containing 20-100 nuclei whereas vaccine strain 'K' and 'low-pathogenicity' variants showed a predominant rounding off of the individual cells and the formation of giant cells containing only 2-5 nuclei.

Fowl Embryo Cell Cultures

The behaviour of pseudorabies virus in fowl embryo

cell cultures has been studied in detail by several European workers, notably in Czechoslovakia. Zuffa and Skoda (1960), Albrecht et al (1963), Masic and Petrovic (1964), Pette and Mahnel (1964) and Kabelik and Korych (1966) showed that the rate of growth of the virus in fowl embryo cells was of the same magnitude as that in mammalian epithelial cells, while Skoda, Brauner, Sadecky and Mayer (1964a) observed that most strains produced a marked CPE consisting of foci of rounded degenerate and refractile cells. The onset and course of the CPE depended on the dose and degree of virulence of the virus. At high multiplicies of infection there was withdrawal of the cytoplasmic processes and rounding off of affected cells as early as 24 hours; and many isolated cells underwent necrosis and desquamated from the cell sheet. Total disruption of the monolayer occurred within the next 24-48 hours. At low multiplicies of infection focal rounding and necrosis of isolated cells did not occur within the first 24 hours while with limiting dilutions of virus the first evidence of CPE was not observed before the 5th or 6th day post infection.

With freshly isolated strains of virus, the focal areas of necrosis were well-defined and destruction of the cell sheet proceeded at a slow pace but with the BUK

strain of fowl embryo cell culture-adapted virus the CPE developed more rapidly and even at limiting dilution it induced total necrosis of the cells within 72 hours. Furthermore, the virus titres obtained with the BUK strain were significantly higher than those of freshly isolated strains.

The ability to induce syncytia and nuclear inclusions in fowl embryo cell cultures seemed to be an attribute of freshly isolated strains (Zhelev and Khristov, 1962; Rusev and Mateva, 1962; Lucas, Metianu and Atanasiu, 1966; Joubert and Bouillon, 1969).

Unlike the syncytia produced in rabbit kidney cells which, according to Kaplan and Vatter (1959), were probably formed by the proliferation of the affected nuclei, the development of syncytia in the later stages of infection in fowl embryo cell cultures was considered by Skoda et al (1964a) to be due to cell fusion. However, after several serial passages in fowl embryo cells the virus apparently loses the ability to produce multinucleate giant cells (Albrecht et al, 1963; Zuffa and Greiglova, 1966).

The size of the "plaques" produced on fowl embryo cells grown under an agar monolayer may depend on several factors including the time of reading, the concentration of agar, the density of the cell layer,

the age of the cells, the technique of inoculation and the individual characteristics of the virus strains (Skoda et al, 1964a). A report by Skoda et al (1964a) that virulent strains tended to produce smaller plaques whereas attenuated strains, obtained after adaption by successive passages in fowl embryo or fowl embryo cell cultures generally induced larger plaques did not support the findings of other workers including Bartha (1961) who failed to find a clear-cut link between plaque size and virulence of the strain. However, most workers are agreed that there is probably a direct linear relationship between the temperature of incubation of the culture and the diameter of the plaques produced; and that higher temperatures generally induce larger plaques. There is no evidence that virulent and attenuated strains differ in this respect, (Mayer and Skoda, 1962; Zuffa and Greiglova, 1966).

SEROLOGY

THE VIRION

Two methods are employed for the visualization of the virus in the electron microscope; the first is by ultracentrifugation of a clarified suspension of infected tissue or cell culture and examination of the resulting pellet by either the negative contrast (Reagan et al, 1952) or the shadow casting techniques (Reissig and Kaplan, 1962) whereas the second method involves the preparation of ultrathin sections of infected cells (Kaplan and Vatter, 1959). Both methods are entirely successful and the literature on the subject has been well reviewed by Lautie (1969).

In negatively-stained preparations the virus particles of Aujeszky's disease appear as regular structures having a central core of DNA surrounded by a protein coat that exhibits icosahedral symmetry. The protein capsid is composed of 162 hexagonal or pentagonal hollow capsomeres and is usually surrounded by a loose limiting membrane. The enveloped virions are approximately 180-250 nm. in diameter and are heat, ether and acid labile (Kaplan, 1966).

The developmental stages of the virus in infected tissues and mammalian cell cultures have been studied by Kaplan and Vatter (1959), Schulze and Benndorf (1963), Felluga (1963), Bendixen and Borgen (1965) and Becker

(1966) who showed that maturation of the virus in infected pig kidney cells is accompanied by profound ultrastructural changes in the nucleus and cytoplasm. In the nucleus, there are masses of marginated chromatin, large dense granules and A-forms of the virus. These A-type virions are characterized by a single dense coat investing a hollow eccentric core and occur either individually or in clusters, or sometimes in crystalline arrays. They are smaller than mature particles and measure about 110 nm in diameter (Felluga, 1963).

Viral forms with a double coat are found both in the nuclear matrix, near the inner nuclear membrane, and in the dilatations of the perinuclear space while viral forms with a triple coat, and measuring about 160-170 nm generally occur in the lumina of the smooth surfaced vacuoles situated in the juxtanuclear zone of the cytoplasm. Other particles having four dense coats have also been demonstrated in the polylamellar systems of the cytoplasm and in the vicinity of the cell wall. The virions with 2, 3 and 4 coats have been referred to as B, C and D forms respectively (Felluga, 1963). Particles similar to the A-forms have also been demonstrated in the capillary endothelial and alveolar lining cells in the lungs of experimentally infected rabbits (Becker, 1966).

DEMONSTRATION OF VIRAL PROTEINS

Surprisingly little information is available regarding the development of virus-specific proteins in pseudorabies-infected cells which can be recognised by complement fixation (CF) and immunodiffusion (ID) tests.

Complement-fixing Antigens: Although the complement fixation antigens of pseudorabies virus have not been characterized, it is probable that the virus precursor proteins demonstrated by Hamada and Kaplan (1965) in pseudorabies-infected cells belong to this class of antigens. Hamada and Kaplan (1965) attempted to identify the proteins synthesized by infected cells by incubating cultures in a medium containing 14_C-labelled amino-acids. These virus-specific proteins were precipitated with antiviral gamma globulins and the amount of radioactivity in the precipitates was taken as a measure of the amount of the antigens synthesized. The findings also showed that, immediately after the infection, the rate of synthesis of cell specific proteins declined and that serologically distinct proteins were formed which bore no precursor relationship with the virus proteins and which were not present in the uninfected cells. This was accompanied by the production of virus-specific proteins. Synthesis of these proteins which reacted specifically with the

antiviral gamma globulins commenced at 3 hours after infection and continued until the end of the growth cycle.

According to Nachkov, Christophorov, Gnenev and Stoyanov (1958) the complement-fixation test in the diagnosis of pseudorabies was first described by Jonnescu and Zavojam (1942) who used an alcoholic extract of brain tissue from rabbits experimentally infected with the virus. Similarly, extracted tissue antigens were found to be useful in the demonstration of CF antibodies in convalescent pig sera (Zcymbal and Culescu, 1953; Nachkov et al, 1958). However, in the hands of Csontos and Romvary (1960) the tissue antigens from infected rabbits, pigs or fowl embryos were unreliable and were often anticomplementary. On the other hand, antigens obtained from infected cell cultures proved to be superior and the reactions obtained were highly specific.

Immunodiffusion Antigens: The application of immunodiffusion tests for the rapid demonstration of specific viral antigens in pseudorabies-infected tissues or cell cultures has not been explored in detail. Bazylev and Fomin (1965) demonstrated specific chloroform resistant antigens in the pancreas and lymph nodes of animals naturally or experimentally infected with Aujeszky's disease virus while Metianu and Sudi (1969) confirmed

the existence of specific viral antigens in the brains and lungs of infected animals. When such antigens are diffused against pseudorabies hyperimmune pig or horse sera or convalescent pig sera two lines of precipitation emerge. Similar antigens have also been obtained from infected mammalian cell cultures some of which appear to be cell-bound and are only released by destruction of the infected cells by alternate freezing and thawing. Antigens prepared from cell cultures infected with virulent strains of the virus generally give three lines of precipitation against a hyperimmune serum. However, one of the antigenic components is lost when the strains are passaged serially in cell cultures and only two lines emerge. The tests are generally conducted at 4°C and the precipitation lines appear within 24 hours.

DEMONSTRATION OF ANTIBODIES

Pigs, monkeys and rarely guinea pigs recovering from pseudorabies infection develop a solid type immunity that is characterized by the presence of virus neutralising antibodies in their blood sera (Shope, 1931; Hurst, 1936; Galloway, 1938). However, there is little published information on the development of antibodies in the more susceptible species of animals such as ruminants, carnivores and fur-bearing animals which do occasionally recover from the disease. Moreover, in most studies,antibodies to pseudorabies have been

demonstrated by the virus neutralisation tests in guinea pigs or cell cultures and the usefulness of complement-fixation and agar gel diffusion tests in the detection of the antibodies has received but little attention.

Complement-fixing antibodies: Nachkov et al (1958) examined the sera of 530 pigs in 16 infected farms in Bulgaria for CF antibodies using antigens obtained from infected rabbit brains. The antibodies were detected in 50 to 70 per cent of animals about 25 to 30 days after the first clinical evidence of infection in the herd. Csontos and Romvary (1960) examined naturally infected pigs and observed that CF antibodies appeared on the seventh day. Peak titres developed at about 5 weeks and the antibodies disappeared 3 to 4 weeks after. The detection of the antibodies in early convalescence was similar to that reported in herpes simplex infection (Buddingh, Schrum, Lanier and Guidry (1953) and was possibly due to the use of cell culture antigens. Anticomplementary factors in the sera were removed by heat inactivation followed by refrigeration overnight after adjusting the pH to a range of 4 - 4.5.

Immunodiffusion Antibodies: In herpes simplex infections antibodies to the several precipitating antigens appear irregularly (Scott and Tokumaru, 1964). However, sera prepared in experimental animals by

repeated inoculations of purified viral antigens emulsified with Freund's adjuvant contain high titres of precipitating antibodies (Tokumaru, 1965a, 1965b, 1970); but similar data are lacking for pseudorabies. The only available report that mentions the presence of precipitating antibodies in convalescent pig sera is that by Metianu and Sudi (1969). Reference sera for precipitation tests were prepared by hyperimmunising horses (Bazylev and Fomin, 1965; Fomin, 1966), or pigs, (Metianu and Sudi, 1969) with several inoculations of live cell culture virus. However, quantitative data are not available.

Neutralising Antibodies: Several large scale serological studies have been carried out in different countries to demonstrate virus neutralising antibodies in the sera of pigs in attempts to trace the origin of outbreaks of pseudorabies in farm stock (Shope, 1935a, 1935b; Carneiro, 1941; Carneiro and Cardim, 1947; Kojnok, 1962) or to determine the incidence and distribution of the disease in endemic or recently infected territories (Lamont, 1947; Divo, Goldman and Lugo, 1951; Mackay et al, 1962; Burrows, 1963, 1966; Borgen and Bendixen, 1965). In one such survey, Heynen (1941) examined 3,000 pig sera from 31 German abattoirs and detected neutralising

antibodies in only a small percentage of samples. Glover (1938) failed to detect antibodies in 250 samples of pig sera from different parts of England and Lamont (1947) reported antibodies in only 1 per cent of 760 bacon pigs in Northern Ireland. However, later surveys conducted in the United Kingdom revealed a much higher incidence in bacon pigs (Mackay et al, 1962) in boars (Burrows, 1963) and sows (Burrows, 1963; McFerran et al, 1966) although the incidence of the clinical disease in Scotland, South East and South West England and Central Wales, appeared to be very low (Burrows, 1966). In Austria, Kubin (1969) observed that a large number of indigenous pigs did not possess antibodies to pseudorabies whereas sera from 21 per cent of slaughter pigs imported from the neighbouring countries did contain antibodies.

In none of these surveys were antibodies to pseudorabies detected in cattle sera (Bendixen and Borgen, 1965; Borgen and Bendixen, 1965; McFerran et al, 1966). Nor did sera from cattle, horses and sheep that survived outbreaks of the disease on six different pig farms in Hungary contain detectable amounts of antibodies (Kojnok, 1962).

The onset of neutralising antibodies in pigs recovering from the natural disease has been discussed

by a few workers including Shope (1935a) who reported that they generally appeared about a month after recovery. However, Berbinschi (1956) noted that antibodies were present in 33 per cent of animals in outbreaks of only 5 - 8 days standing, while after 5 weeks all the animals had antibodies in their sera and in some the titres reached high levels. Also, Johnston et al (1961) noted that antibodies developed within 14 to 27 days in a litter of pigs, whose dams had antibodies in their sera. In Akkerman's (1963) survey traces of neutralising antibodies were detected at 4-7 days and the titres which increased by the 10th to 17th day probably persisted throughout life. Skoda et al (1963) found great variations in the persistence of neutralising antibodies in convalescent pigs. In some animals they persisted for 54 months whereas in others antibody titres were barely detected at 3 months. There is a lack of information regarding passively-derived antibodies in the new-born pigs but Akkermans (1963) demonstrated colostrum-derived antibodies at 48 hours after birth which persisted at high titres for periods of 3 to 8 weeks. Sows vaccinated in late pregnancy with two doses of live attenuated vaccine transferred neutralising antibody to their offspring which persisted for 6 to 14 weeks (Zuffa, 1965).

The development of neutralising antibodies in vaccinated pigs, cattle and sheep has received some attention. In piglets, inactivated vaccines fail to induce neutralising antibodies even when given in 2-3 doses of 2.5 to 10 ml. each (Zuffa, 1963a) whereas, following a single dose of live attenuated fowl embryo cell cultured vaccines, poor to moderate titres develop (Zuffa, 1963b, 1964). Antibody titres increase substantially after the injection of a second dose of vaccine and Zuffa (1963b) reported titres ranging from 1:32 to 1:512 by this method. In the studies of Bran et al (1969) the neutralising index values in the secondary response ranged from 3.7 to 4.3 compared with values of 0.6 to 1.1 following the primary response. A similar picture emerged in vaccinated cattle (Zuffa, 1963b; Zuffa and Dlhy, 1964; Kojnok, 1964) and dogs (Kojnok, 1964).

In sheep, neutralising antibodies were either not detected (Bognar and Kucsera, 1966) or were detected in low titres after injection with a single dose of a live vaccine (Zuffa, 1963b, 1966) while in another study, only 10 per cent of 110 sheep developed antibody titres after a single inoculation of live vaccine. With a second dose of the vaccine, sero-conversion was 100 per cent (Kucsera, 1966) until at 5 months the titres

gradually fell and only 17 per cent of the animals had antibodies. Similar data regarding the persistence of neutralising antibodies were furnished by Nedyalkov et al (1968) who inoculated sheep with 2 doses of different types of inactivated vaccines.

Very little information exists regarding the development of neutralising antibodies in experimental laboratory hosts. Zuffa (1963a) obtained moderate to high titres of antibodies in rabbits, guinea pigs and mice, following repeated injections of formaldehyde inactivated or ultraviolet-irradiated vaccines and high titres were induced in fowls by live virus only.

A pseudorabies virus-neutralisation test in guinea pigs was developed by Shope (1931) to test the pathogenicity of residual virus in virus-serum mixtures. The test proved to be reliable and was useful in several experimental studies and serological surveys. (Shope, 1935a, 1935b; Carneiro, 1940; 1941; Heyman, 1941; Carneiro and Cardim, 1947; Lamont, 1947). A few investigators claimed success with the rabbit as the indicator host (Manninger and Marcis, 1937; Hirt, 1937), but others obtained only irregular results (Shope, 1931; Sabin, 1934a; Glover, 1939). Although mice and fowl embryos were used as alternative hosts by some workers (Glover, 1939; Kojnok, 1962; Akkermans, 1963) the use of

laboratory animals has now been replaced almost entirely by cell cultures.

The standard procedure for cell culture neutralisation tests is to incubate equal volumes of two-fold serial dilutions of the test sera and 100 $TCID_{50}$ doses of the virus suspension for one to two hours either at room temperature (Horvath, 1959; Johnston et al, 1961; Mullaney and Murphy, 1962; Reichel, 1964) or at 37°C (Kaplan and Vatter, 1959; Kojnok, 1962; Skoda and Zuffa, 1962; McFerran et al, 1966) and to test the mixtures for virus by inoculating them into mammalian or fowl embryo cell cultures. The literature on the subject has been well reviewed by Lautie (1969).

EPIZOOTIOLOGY

THE PIG RESERVOIR

In its natural state Aujeszky's disease attacks several species of mammals but its chief reservoir is the pig (Shope, 1942; Jivon, 1956; Kojnok, 1962). For many years the pig was not suspected in the transmission of the disease until Ratz (1914) and other investigators (Shope, 1934, 1935a; Koves and Hirt, 1934; Burggraaf and Lourens, 1932; Steiner and Lopes, 1935) observed that while the disease in pigs was generally symptomless and usually ran a mild course it was, nevertheless, contagious. They also noted that healthy swine housed on the same premises as infected pigs developed the disease after an incubation period of 5 days or longer (Koves and Hirt, 1934) and they further suggested that the likeliest vehicles of transmission were the infective bronchial secretions, saliva and urine. However, it was Shope (1935a) who first furnished experimental evidence of the contagiousness of the pig disease and, by using rabbits as the indicator host, showed that the portal of entry and exit of the virus was the nasal mucosa. Virus was not demonstrable in the salivary glands, urine and the faeces. Shope's findings were soon confirmed by other workers including Lyukashev and Rotov (1939) and Manninger (1939). Some years later Kojnok and Greczi (1957), Taga et al (1957),

Nikolitsch (1958), Akkermans (1963), McFerran and Dow (1964, 1965) Corner (1965) and Kojnok (1965) observed that the infection was more frequently established by intranasal inoculation than by other parenteral routes, with the possible exception of the more drastic intracerebral route, while Ghenev and Stoyanov (1958) and Kojnok, Glah and Surjan (1959) induced a fatal respiratory syndrome in pigs by the intratracheal inoculation of virulent strains of virus. Numerous reports of the isolation of Aujeszky's disease virus from the tonsils (Berbinschi, 1962) trachea and lungs (Szent-Ivanyi, 1962; Csontos, 1964; Bodon et al, 1968) and the bronchial lymph nodes (Bodon et al, 1968) of naturally infected pigs, and experimental evidence of the persistence of the virus in the internal organs of apparently healthy pigs (Persev, 1958; Surdan, 1958 cited by Nikitin, 1961; Nikitin, 1961) have strengthened the hypothesis that swine are the natural carriers of the virus.

The work of Kojnok (1957, 1965) may be regarded as a signal contribution to the better understanding of the epizootiology of the disease. Piglets suckled by apparently normal sows developed Aujeszky's disease and virus was demonstrated in the milk of the dams and the gastric contents of the dying piglets. More recently

Kojnok (1965) also demonstrated that clinically healthy and "serologically immune" sows carry the virus in the upper respiratory tracts since he was able to recover virus from the nasopharynx of 3.4 per cent of animals. Furthermore, Wilke and Dannenberg (1968) reported the virus in the tonsils, lungs and muscle tissue of 4.1 per cent of apparently healthy pigs in endemically infected farms, despite the fact that 86.5 per cent of the pigs had virus neutralising antibodies in their sera; and Nikitin (1961) isolated the virus from the lungs and liver of a pig 186 days after clinical recovery. Thus, the mild and contagious character of the infection and the recoveries of the virus from the respiratory tract of pigs, even in the presence of circulating virus-neutralising antibodies, are in support of the current hypothesis that the pig is the natural reservoir host of pseudorabies virus.

THE RODENT RESERVOIR

The role of rodents in the epizootiology of Aujeszky's disease was first postulated by Balas (1908), Hutyra (1910) and Schmiedhoffer (1910) and later discussed by several investigators mostly on the basis of circumstantial data (Ratz, 1914; Burggraaf and Lourens, 1932; Lourens, 1935; Gerlach and Schweinburg, 1936; Becker, 1939; Vianello, 1942; Toneva, 1958). The

massive exodus of rats (Nikitin, 1953) or their disappearance from infected farms (Lamont and Kerr, 1939; Janowski and Oberfeld, 1965) and the detection of sick rats or rat cadavers (Gordon and Luke, 1952; Lyubashenko, et al, 1958; Bartosz, 1962) on farms shortly before or during the course of the disease in farm animals were considered as indirect evidence of the presence of the disease in the rat population. The literature also contains reports of alleged but unproven outbreaks of the disease in wild rats (Ledyev and Rakhmanov, 1964; Skoda and Grunert, 1965).

The first direct evidence of the disease in rats stems from Hutyra's (1910) report which clearly showed that the virus was present in the brains of rats found dead on farms where Aujeszky's disease had attacked cattle. Similarly, Lukashev and Rotov (1939) recovered the virus from the internal organs of field mice found dead in an Ukranian piggery where the disease was known to occur. A few other workers have isolated the virus from naturally infected rats (Tepper, 1957; Nikitin, 1960; Becker and Herrmann, 1963) and, of these, Nikitin's report is the most comprehensive to date.

From the available data on the natural and experimental disease in rats, it is possible to draw some conclusions regarding the reservoir role of this

species. Rats can be infected per os (Ratz, 1914; Burggraaf and Lourens, 1932; Shope, 1935b; Nikitin, 1960; McFerran and Dow, 1970) and the infection is probably dose-dependent. For instance, Becker and Herrmann (1963) observed that adult rats of different ages were not infected whereas the two mothers which had eaten the carcases of intracerebrally infected offspring, succumbed to the infection. Likewise in Nikitin's experimental study of 1440 wild brown rats (Nikitin, 1960) the mortalities appeared to be dose-related. Surviving rats carried the virus in their brains, livers and spleens for periods up to 80 days and in the lungs and urinary bladders for approximately 130 days.

Occasionally the rat disease is symptomless and, as Nikitin emphasized, the natural disease in rats is generally enzootic although it may, sometimes, assume epizootic proportions and that the virus may persist between outbreaks in the survivor rats. There is also a good deal of circumstantial evidence to suggest that rats may be infected by pigs and that the reverse can also occur.

The nature of the carrier state in the rat is intriguing and Becker and Herrmann (1963) regarded it as a true form of latency. Unfortunately, serological data from carrier rats are lacking and it is not

possible, therefore, to compare the relative roles of the rat and the pig in the mechanisms of survival of the virus.

TRANSMISSION AMONG PIGS

Shope (1935a) demonstrated that the spread of the disease from pig to pig was by way of the nasal passages since, in 12 out of 14 experiments, healthy pigs contracted the infection from subcutaneously and intramuscularly infected pigs after an incubation period of 3 to 11 days. Virus was detected in the nasal washings of the infected swine not only during the incubation period and throughout the course of the illness but also on the first four days of convalescence. Similarly, in the experiments of McFerran and Dow (1964, 1965) and Sabo et al (1969) the virus was detected in high titres in the oral and nasal secretions of intranasally infected pigs from 24 hours post-infection and persisted therein for about a fortnight.

In the studies of Sabo (1969) weanling pigs first immunized by subcutaneous vaccination with the attenuated BUK strain and then challenged per os with virulent virus developed no clinical symptoms but maintained the virulent virus in the throat mucosa for periods up to 20 days; the neutralising antibody titres on the day of challenge ranged from 1:8 to 1:16. These

findings are highly important because they emphasize the danger of regarding animals with antibody titres as being free of the infective virus.

The role of faeces and urine in the transmission of the disease merits comment. Koves and Hirt (1934) and Vianello (1942) claimed to have recovered the virus from the urine of naturally infected pigs while Lukashev and Rotov (1939) demonstrated the virus in the urine of subcutaneously infected pigs. Although the detailed investigations of Andrev and Uzunov (1940) and Sabo et al (1969) showed that the kidneys, sometimes, harboured the virus, other workers, including Shope (1933), Andrev and Uzunov (1940) and McFerran and Dow (1964), reported that the urine and faeces were never infective. On the other hand, the frequency of virus recovery from the urine of experimentally infected rabbits and gray rats (Andrev and Uzunov, 1940) and from naturally infected sheep (Bogdon, 1961) probably reflect differences in the virus strains and in the responses of the different species of animals.

Transmission through milk is also a possibility (Nikitin, 1949; Kojnok, 1957) and even venereal spread was suspected by Akkermans (1963) because boars from disease-free farms developed antibodies to Aujeszky's disease after inseminating sows from other farms,

seventeen per cent of which had virus-neutralising antibodies. Akkermans postulated that the external genetalia of the male became infected during coitus by the nasal secretions of carrier sows and while she succeeded in isolating the virus from the prepuce and vagina of experimentally infected animals she found no evidence of virus secretion in the seminal discharges of the boars.

TRANSMISSION IN OTHER SPECIES

Pseudorabies in cattle, sheep, cats, dogs, captive foxes, mink and ferrets is a well defined clinical but non-contagious disease (Shope, 1931; Shahan et al, 1947b; Lyubashenko et al, 1958), which has no seasonal incidence (Shope, 1942). Most outbreaks are sporadic (Szilard, 1927; Bang, 1932; Nicolic, 1932; Jonnesco, 1934; Cordier and Menager, 1937; Johnston et al, 1961; Dow and McFerran, 1964) but, on occasion, the disease in cattle and sheep flares up and may assume epizootic proportions (Marcis, 1933; Berecz, 1961; Kojnok, 1962; Saunders and Gustafson, 1964; Ivanov, Kharalambi and Stamenov, 1968).

The majority of cases in cattle have been traced to contact with either apparently healthy or overtly infected pigs (Burggraaf and Lourens, 1932; Koves and Hirt, 1934; Shope, 1935a; Hoyt, 1946; Toneva, 1958) and

in the early American farm literature there is well-documented circumstantial evidence of "mad-itch" having occurred in cattle that had eaten green corn while at pasture with hogs (S.B.D., 1823; Morgan, 1855; McIntosh, 1895). Undoubtedly, the establishment of the disease in the Midwest corn belt in the U.S.A. followed the practice of grazing swine with cattle (Hanson, 1954). Similarly, outbreaks in sheep (Marcis, 1933; Koves, 1935; Kojnok, 1962; Ivanovics et al, 1968) and dogs (Mullaney and Murphy, 1962; Dow and McFerran, 1963) were traced to contact with infected pigs.

There are several authenticated reports of the disease first manifesting itself in mixed farms in pigs and then spreading to cattle (Lukashev and Rotov, 1939; Borgen and Bendixen, 1965), sheep (Kotov, 1950; Kojnok, 1962) and dogs and cats (Kotov, 1950; Becker, 1961). Likewise, when cattle were moved to fattening pens and reared with pigs the disease appeared within a few days. (Anon, 1956). The hypothesis that the disease generally originates in the pig and is transmitted to other stock is also supported by serological data. Surveys carried out in several countries have shown a moderate to high incidence of virus-neutralising antibodies in the sera of bacon pigs, sows and gilts (Shope, 1935b; Carneiro, 1941; Heyman, 1941; Lamont,

1947; Mackay et al, 1962; Divo et al, 1951; McFerran et al, 1966; Burrows, 1963, 1966; Dragonas et al, 1969) but none in the sera of cattle (Shope, 1935a; Bendixen and Borgen, 1965; Borgen and Bendixen, 1965; McFerran et al, 1966).

However, on farms where affected cattle have had no direct contact with pigs, it is possible that the infection was acquired from rats (Tepper, 1960) or, as has been suggested by Lamont (1947), by rubbing against contaminated fomites. The abrasions produced on the skin probably facilitate penetration of the virus into the underlying tissues.

There are no published reports, at present, of the minimum infective dose of the virus for cattle, but recent studies by Dow and McFerran (1966) suggest that very small quantities of virus are sufficient to initiate the infection in sheep. Since the natural and experimental disease in cattle and sheep have similar clinical features and mortality patterns, it is reasonable to suppose that there is little difference in the susceptibility of the two species to infection through the skin and other natural routes. It is also highly likely that ruminants can contract the infection from fomites contaminated with infective discharges from pigs or rodents. Solomkin and Tutushin (1956) and

Ustenko (1958) reported that on infected farms the virus of Aujeszky's disease survived on grains, hay, straw, sacks and wooden planks for 10-30 days in summer and up to 46 days in winter; but quantitative data were not given.

In cats and dogs, Aujeszky's disease is generally transmitted by pigs (Kotov, 1950; Estola et al, 1965; Canic, 1969; Huck et al, 1969) and rats (Balas, 1908; Cassels and Lamont, 1942) but a few outbreaks have also stemmed from infected pork, (Costa, 1951; Ercegovac et al, 1958; Becker, 1961; Stepenko, 1962; Horvath and Papp, 1967). Similarly, epizootics of the disease in captive foxes (Ugorski, 1958; Janowski, Janowska and Wijaska, 1965; Bitsch, Knox and Munch, 1969) and mink (Vanek, Groch and Sanda, 1962; Christodoulo et al, 1970) have been traced to infected pig scraps.

OBJECTIVES

It is said that the clinical expression of disease is a product of the host-parasite interaction. The main objective of this present work is to elucidate the latter through investigations of the responses of laboratory-reared chickens, mice and rats exposed to Aujeszky's disease virus. From the review of the available literature it is clear that rats and mice are known to be naturally and experimentally susceptible to Aujeszky's disease and that chickens can be experimentally infected, but quantitative data are lacking, and no systematic investigation appears to have been made in chickens. In attempting to fill this gap in our knowledge of the pathogenicity of the virus, it was realised that a detailed study of the basic parameters such as the effect of age, dose of virus and route of inoculation and also the distribution of virus in different tissues of experimentally infected chickens would be helpful in understanding the behaviour of the virus in this supposedly unresponsive host. In addition, the findings might shed some light on the epizootiology of the disease since it is known that in endemic areas poultry remain unaffected even when raised on the same premises as susceptible farm livestock. Moreover, as rats and mice are known to be susceptible to the experimental infection, it was thought that a comparison of the responses of

rodents and chickens to the virus might also provide useful information as to the behaviour of the virus in ecologically unrelated hosts.

As an adjunct to the study of experimental pathogenicity of Aujeszky's virus for chickens, mice and rats, it was decided to ascertain the relative susceptibilities of different cell cultures to the virus with the objective of developing an in vitro indicator system. Such a system would assist in correlating the data of clinical responses to experimental infection with those of virus recovery. It was also felt that comparison of the infectivity of the virus to experimental hosts and cell cultures would greatly assist in determining whether young chicks, mice and rats could be used as alternative indicator systems for the isolation of virus from suspect clinical cases of Aujeszky's disease.

MATERIALS AND METHODS

VIRUS STRAINS

The three strains of Aujeszky's disease virus used in this study were designated as the McFerran, Hungarian and Weybridge strains.

McFERRAN STRAIN

This strain, which was originally isolated in rabbit kidney cell cultures from a natural case of Aujeszky's disease in a pig (McFerran and Dow, 1965), was received by courtesy of Dr. McFerran, Stormont, Belfast. In the present investigation, the virus was maintained in a pig kidney cell line (PK15) and most studies were carried out with this strain.

HUNGARIAN STRAIN

This strain of reduced virulence was initially isolated and propagated in pig kidney cells by Bartha (1961). Later it underwent several serial transfers in fowl embryo cells and was used as a vaccine strain (Skoda, 1962). A culture of this strain was kindly supplied by Professor Dow, Stormont, Belfast and was maintained during this present investigation, in fowl embryo fibrobalsts and was used for comparative studies.

WEYBRIDGE STRAIN

This was a recent field isolate which was supplied as infective brain tissue by courtesy of Dr. Shiela Cartwright, Central Veterinary Laboratory, Weybridge. The virus was passaged in pig kidney cells (PK15) before

it was used for pathogenicity studies in experimental chicks.

CELL CULTURES

PREPARATION OF PRIMARY CULTURES

Kidney Cultures: Monolayer cultures of renal epithelial cells from the calf, ferret, lamb, piglet and pup were prepared according to the multiple extraction procedure of Younger (1954), with minor modifications. The kidneys were washed free of blood with sterile Earle's[1] or Hank's[2] balanced salt solution (B.S.S.) or phosphate buffered saline (P.B.S.) prewarmed to 37°C and containing 500 units, 500 µg and 20 µg per ml., respectively, of penicillin[3], streptomycin sulphate[3] and nystatin[4] or fungizone[4]. The kidney cortical tissue was minced with scissors, thoroughly washed in Earle's or Hank's B.S.S. and transferred to a trypsinization flask. One hundred ml. of 0.05 per cent trypsin[5] solution in P.B.S. (pH 7.2) were added and the suspension was stirred for 20 to 30 minutes at room temperature (20 to 22°C). The tissue fragments were allowed to settle and the supernatant fluid was discarded. One hundred ml. of fresh 0.05 per cent trypsin solution were added to the tissue and the suspension was stirred by a magnetic stirrer for 45

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1 Earle, W.R. 1943.

2 Burroughs Wellcome & Co. London, Wellcome Research Laboratories, Beckenham, England.

3 Glaxo Laboratories Ltd., Greenford.

4 E.R. Squibb & Sons, Speke, Liverpool.

5 Difco Laboratories, Inc., Detroit, Michigan, U.S.A.

minutes at room temperature. Trypsinization was repeated two or three times and the harvests between the cycles were stored on ice in a flask containing 50 ml. of outgrowth medium supplemented with 10 per cent inactivated calf serum and an antibiotic mixture consisting of penicillin, streptomycin and nystatin or fungizone. If necessary, the tissue remnants were subjected to overnight trypsinization according to the method of Bodian (1956) using an 0.05 per cent of trypsin solution.

The cell suspensions were filtered through sterile gauze and centrifuged at 600 r.p.m. for 15 minutes and the cell pellets were resuspended in 100 ml. of the outgrowth medium and recentrifuged at 1,000 r.p.m. for 10 minutes. After determining the viable count the cells were dispensed into suitable culture vessels so as to contain approx. 1 x 10^6 cells per ml.

Chicken kidney cells: Chicken kidney primary cell cultures were prepared according to the method recommended by Churchill (1965). This consisted of multiple cycles of trypsinization of the minced kidney tissue with an 0.25 per cent solution of trypsin in P.B.S. at 37°C, each cycle lasting for 3 to 5 minutes. The first one or two harvests containing blood elements were discarded and the cells harvested from each

subsequent run were then mixed with a small amount of inactivated calf serum and stored in the refrigerator. Finally, the harvests were pooled, lightly centrifuged and the cell pellet was resuspended in outgrowth medium to contain 2 to 4 x 10^6 viable cells per ml.

Fowl Embryo Fibroblasts: In general, 9 or 10 days old chicken embryos were used for the preparation of fibroblast cultures but in one experiment embryos incubated for 5 to 19 days were used in order to study the pathogenicity of Aujeszky's disease virus on fibroblast cultures obtained from embryos of different ages.

Embryos harvested aseptically were freed of heads, entrails and limbs and the residual tissues minced with scissors. The tissue fragments were washed several times in Earle's B.S.S. or P.B.S. and then stirred with either an 0.1 per cent solution of trypsin for one hour at room temperature or subjected to the multiple extraction procedure using an 0.25 per cent trypsin solution. In the latter case, the cells were harvested at intervals of ten minutes until no more cells were released from the tissues. The rest of the procedure for obtaining the cell suspensions and dispensing the cells into culture vessels was similar to that described for mammalian cell cultures.

CELL LINES

Apart from primary and secondary cell cultures, various types of cell lines were also used during this investigation including the bovine (MDBK)[1], ovine (MDOK)[1] and porcine (Stice - PK- 2a) kidney cell lines which were generously provided by Dr. Madin, Berkeley, California. Cultures of continuous lines of dog kidney (MDCK)[1], baby hamster kidney (BHK21)[2], monkey kidney (LLC.MK-2), rabbit kidney (RK13), porcine kidney (PK15) and human HeLa cells were obtained by normal purchase[3].

Subcultivation of Cell Cultures: The outgrowth medium was decanted and the monolayer rinsed with P.B.S. prewarmed to 37°C. For stripping cells from glass surfaces either 0.25 per cent solution of trypsin or a versene-trypsin mixture containing 0.01 per cent each of versene and trypsin were used. When trypsin was used, it was left in contact with the monolayer for about a minute and then removed. The cultures were then incubated in an inverted position at room temperature or 37°C for 10 to 15 minutes. A small amount of outgrowth medium was added and the cells were

1 Madin, S.H. and Darby, N.B. (1958) Proc.Soc.exp. Biol. N.Y., 98, 574.

2 Macpherson, I.A. and Stoker, M.G.P. (1962) Virology, 16, 147.

3 Flow Laboratories Ltd., Ayrshire.

detached by gentle shaking. Disaggregation of the cells was ensured by gentle pipetting of the suspension. The suspension was centrifuged at 1,000 r.p.m. for 5 minutes to sediment the cells.

When a versene-trypsin mixture was used, the monolayers were incubated with 5 to 10 ml. of the mixture at 37°C for 15 to 30 minutes. Cells were dispensed into culture vessels at a cell density of 1 x 10^5 per ml.

Culture Vessels: Cells were grown either in Roux flasks, 4 oz. flat medicine bottles, Leighton tubes or in standard 'Pyrex' cell culture tubes (6" x $\frac{2}{3}$"). The tube cultures were incubated in stationary racks or on roller drums.

CELL CULTURE MEDIA

Most primary cell cultures and cell lines were propagated in an outgrowth medium containing either Eagle's[1] or Earle's[2] basal medium enriched with 10 per cent heat-inactivated calf serum obtained from the local abattoir. An antibiotic mixture consisting of penicillin, streptomycin and nystatin or fungizone to a concentration of 200 units, 200 micrograms and 10 micrograms per ml. respectively and sodium bicarbonate

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1 Contains 10 per cent tryptose phosphate broth in Eagle's 59 B.S.S. with 0.044 per cent sodium bicarbonate.

2 Contains 0.5 per cent lactalbumin hydrolysate and 0.1 per cent yeast extract (Difco) in Earle's B.S.S. with 0.08 per cent sodium bicarbonate.

at a final concentration of 0.35 per cent were added whereas for LLC-MK2 cells and fowl embryo cells an outgrowth medium containing 5 per cent inactivated calf serum was preferred. In the case of MDCK cells 10 per cent foetal calf serum was necessary to stimulate growth. For chicken kidney cells, the outgrowth medium consisted of Hank's[1] B.S.S. as base, supplemented with 0.5 per cent lactalbumin hydrolysate[2] and 10 per cent inactivated calf serum.

The cultures were maintained in Eagle's, Earle's or Hank's basal media containing 2 to 5 per cent inactivated calf serum, except for fowl embryo cells which were maintained on EYL medium without added serum. The bicarbonate concentration in the maintenance medium varied from 0.08 to 0.16 per cent. Growth and maintenance media were replaced generally twice a week, but with some cultures more frequent refeeding was necessary.

PRESERVATION OF CELL LINES

Trypsinized and versene-dispersed cells were preserved according to the procedure described by Dougherty (1962), with slight modifications. This was done by mixing nine parts of the cell suspension in outgrowth medium containing 20 per cent inactivated

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1 Burroughs Wellcome & Co., London.

2 Micro-Bio Laboratories Ltd., London.

calf or foetal calf serum with 1 part of dimethyl sulphoxide (DMSO)[1]. The mixture was cooled step-wise to freezing point over a period of about 2 hours and then transferred to a deep-freeze cabinet at -65°C.

Cells were resuscitated by rapid thawing in a 37°C water-bath and were dispensed into culture vessels containing outgrowth medium; the medium was changed on the following day.

CYTOLOGY

For routine cytological examination infected and uninfected coverslip cultures harvested at different intervals after infection were fixed in a suitable fixative such as Bouin's fluid, methanol-acetic acid mixture (5 per cent glacial acetic acid in methanol) or Carnoy's fluid, for 10 to 15 minutes and stained with haematoxylin-eosin or Giemsa's stain. The stained cultures were differentiated by passing through ascending grades of ethanol or acetone and acetone-xylene, cleared in 2 or 3 changes of xylene and mounted in DePex[2] mounting medium.

ACRIDINE ORANGE FLUORESCENCE

After fixation in ethanol, infected and uninfected cell cultures were stained with 0.05% acridine orange

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1 The British Drug Houses Ltd., Poole, England.

2 G.T. Gurr & Co., London.

(CL 788) in acetate-HCl buffer, pH 2.7 for 30 minutes. (Negroni, 1964). The cultures were rinsed rapidly in several changes of buffer (pH 7.4) and mounted in 10 per cent glycerol in P.B.S. The specificity of RNA and DNA fluorescence was assessed by examination of the stained coverslip cultures with a Carl Zeiss fluorescence microscope employing a HBO 200W high pressure mercury bulb as a source of ultraviolet light. For fluorescent photography various combinations of exciter and barrier filters were used and photomicrographs were taken on High Speed Ektachrome Film (Daylight) ASA160;23DIN.

PRODUCTION OF VIRUS

STOCK POOLS

Two to 3 days old confluent monolayer cultures of pig kidney cells (PK15) were infected with about 25 ml. of 10^7 to 10^8 $TCID_{50}$/ml. of the McFerran or Weybridge strains of virus and absorption was allowed to proceed at 37°C for 2 hours. The inoculum was then discarded, maintenance medium was added and the cultures were incubated at 37°C. At the height of the cytopathic changes, the cell sheets were detached from the glass surface by gentle shaking or with a glass rod and, together with the culture fluids, were either homogenized in an M.S.E. ultrasonic disintegrator[1] for 2 minutes or frozen and thawed thrice. One or 2 days old fowl embryo fibroblast cultures infected with the Bartha strain were likewise treated when the cell sheet manifested maximum CPE. The suspensions were clarified by light centrifugation and the supernatant fluids were then distributed in small aliquots and stored at -65°C.

VIRUS ASSAY

The infectivity titres of the strains of virus propagated in different primary cell cultures and cell lines were estimated in PK15 cells as follows. The test pool was diluted in maintenance medium in 10-fold series

1 M.S.E. Ultrasonic Disintegrator, 60 watt model. Measuring and Scientific Equipment Ltd., London.

and each dilution of the virus was inoculated in 0.2 ml. volumes into 4 to 5 tube cultures each containing 0.8 ml. of maintenance medium. The cultures were incubated at 37°C overnight in a stationary position and then on the roller drum. The development of the CPE was assessed visually and $TCID_{50}$ titres were calculated according to the method of Karber (1931) or Reed and Muench (1938). The standard errors of the $TCID_{50}$ values were estimated by Pizzi's (1950) method.

PRODUCTION OF VIRAL ANTIGENS

CELL CULTURE ANTIGENS

To detect the presence of specific complement-fixing (CF) and immunodiffusion (ID) antigens, infected cell cultures were concentrated by physical methods. Roux flask cultures of PK15, RK13, MDCK and fowl embryo cells were infected with 25 ml. of 10^8 $TCID_{50}$ per ml. of the McFerran strain of virus. The infected cells and culture fluids were harvested at 24 or 48 hours post-infection at the height of the CPE. The cells were disrupted by sonication or by three alternate cycles of freezing and thawing and the suspension was clarified by centrifugation at 3,000 r.p.m. for 20 minutes. The supernatant fluid was concentrated about 50 to 100 times by forced dialysis against Carbowax (Polyethylene glycol) at 4°C and then dialysed against cold 0.15M NaCl solution. The preparation contained both CF and ID antigens.

In other trials, the crude suspensions obtained by sonication or freezing and thawing were treated with a saturated solution of ammonium sulphate to obtain a final concentration of 35 per cent of the salt. Precipitation proceeded at 4°C for 2 to 4 hours and the precipitate was sedimented by centrifugation at 5,000 r.p.m. for 20 minutes at 4°C. The supernatant fluid was dialysed against 20 to 50 volumes of distilled water

at 4°C for 24 to 48 hours and then concentrated to the original volume by dialysis against Carbowax. The precipitate was dissolved in a small volume of distilled water and dialysed against 0.15 M NaCl solution for 18 to 24 hours at 4°C. The two preparations were then tested for CF and ID activities.

In the studies designed to determine the relative concentrations of cell-bound and released CF and ID antigens, confluent monolayers of PK15 cells were infected with the McFerran strain of virus and the culture fluids were harvested at 24 and 48 hours. The CPE was generally well-marked at 48 hours. The pooled fluids were concentrated about 100 times by dialysis against Carbowax and the crude preparation was further dialysed against 0.15 M NaCl solution. The infected cells were scraped from the glass surface, suspended in a small amount of Hank's B.S.S. and centrifuged at low speed. The cell sediment was resuspended in 5 to 10 ml of Hank's B.S.S. and sonicated. The two preparations were titrated for CF and ID antigens.

TISSUE ANTIGENS

Precipitation Techniques: Twenty per cent suspensions of heart, liver, spleen, lung, kidney, brain and spinal cord from chicks and rats experimentally infected with the McFerran strain of virus were prepared in chilled

Eagle's B.S.S. and the suspensions clarified by centrifugation at 3,000 r.p.m. for 20 minutes. The supernatant fluids were inactivated at 56°C for 1 hour and dialysed against 0.15 M NaCl solution for 24 hours at 4°C. Any precipitate that formed was removed by centrifugation. The supernatant fluids were precipitated with saturated ammonium sulphate solution and the solid and fluid phases were harvested as described previously and tested for CF and ID antigens.

Negative control antigens were prepared similarly from uninfected tissues.

EXPERIMENTAL ANIMALS

CHICKENS

The chickens were mainly of the Leghorn breed and were hatched and reared in the laboratory. They were used at various ages and were inoculated by different parenteral routes. Blood samples for sera were collected from the wing or jugular veins but for virus recovery, the blood was collected in vials containing "sequestrene"[1] to which an antibiotic mixture of penicillin (500 units), streptomycin (500 μg) and fungizone (20 μg) was previously added.

FOWL EMBRYOS

Fertile eggs, mostly from White Leghorn hens were obtained from the Edinburgh College of Agriculture, Easter Howgate Farm and were incubated in the laboratory.

MICE

These were a laboratory-bred stock of mixed population and were inoculated by different routes as one-day, one week and 8 to 11 week old animals.

RABBITS

Young European rabbits of unknown breeds were used. They were injected either by the subcutaneous or intramuscular or intravenous routes and bled for serum from the marginal veins of the ear.

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1 Stayne Laboratories Ltd., High Wycombe, Bucks.

RATS

The rats belonged to a laboratory-bred stock of Carworth Europe random-bred rats of Wistar origin (C.F.H.B.). They were of 9 to 10 weeks of age and weighed on an average 250 grams and were inoculated by diverse routes.

SERA

PIG ANTI-AUJESZKY'S DISEASE SERUM

Two serum samples were used in this investigation. The first, which was kindly supplied by Dr. McFerran, was prepared in a pig which had received four injections of Aujeszky's virus. The second was obtained from a six weeks' old pig which was challenged intranasally with the virus following clinical recovery from experimental infection.

RABBIT ANTI-AUJESZKY'S DISEASE SERUM

Two rabbits were inoculated by both the intravenous and intramuscular routes with 2.0 ml. of the McFerran strain of virus (10^8 $TCID_{50}$/ml) that was inactivated at 56°C for 60 minutes. For intramuscular inoculation, the inoculum was emulsified with equal amounts of Freund's incomplete adjuvant[1] and the suspension was injected into multiple sites. One week later, the animals were similarly reinoculated intravenously and intramuscularly. Serum samples were obtained at weekly intervals up to 2 months post-infection.

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1 Difco Laboratories, Detroit, Michigan, U.S.A.

VIRUS ISOLATION

Specimens of brain, spinal cord, heart, lung, liver, spleen and kidney and, in some instances, testis, adrenal gland and muscle tissue from the site of inoculation were collected at necropsy.

On a few occasions, urine and blood samples were also collected at different intervals post-inoculation. The tissues were emulsified in sterile sand by means of a mortar and pestle or a Griffith's tube, maintenance medium containing antibiotic mixture being added to give a 20 per cent suspension. Samples of throat and cloacal swabs collected from some of the experimentally infected chickens were emulsified into small amounts of maintenance medium and the contents were frozen and thawed thrice to liberate intracellular virus. Infective suspensions were centrifuged at 3,000 r.p.m. for 20 minutes and the supernatant fluids were then inoculated in 1 ml. amounts into four PK15 culture tubes per suspension. After absorption for 1 to 2 hours at 37°C the inocula were discarded and the monolayers washed thrice and replenished with maintenance medium. Pooled samples of blood and urine were similarly inoculated.

In experiments designed to ascertain the virus concentration in the infective tissues, the infective suspensions were diluted in ten-fold series in maintenance medium and the dilutions were inoculated in 0.2 ml

amounts into 4 to 5 tissue culture tubes each containing 0.8 ml. of maintenance medium. The cultures were incubated at 37°C and examined daily for 7 days. The virus was detected by its characteristic CPE and identified when necessary by neutralisation with specific antiserum. In cases where no CPE was detected, three serial passages were carried out before the specimen was regarded as being negative. When an infective suspension was toxic to the cell culture, it was appropriately diluted in the maintenance medium and the test was repeated reducing the period of absorption of the virus to 1 hour at 37°C.

ELECTRON MICROSCOPY

To establish the identity of the virus used in these studies morphological confirmation was sought by electron microscopy.

The electron microscope used was the model AEI-EM-6B[1] and a 2 to 4 per cent aqueous solution of phosphotungstic acid[2] (pH 6 to 7) was employed for negative staining.

METHOD

Infective cell culture fluids and tissue suspensions were first clarified by centrifugation at 5,000 r.p.m. for 30 minutes and the supernatant fluids were centrifuged again at an average speed of 140,000 g for 2 hours in an angle High Speed 'Omikron' centrifuge[3]. After suspending the pellets in 2 drops of distilled water one drop of the suspension was then mixed with one drop of the stain. A carbon-coated collodion grid was then gently lowered face downwards on the surface of the stained suspension and after 30 seconds, the grid was lifted and excess fluid was removed by careful blotting. After allowing it to dry, the grid was screened in the electron microscope, using an initial magnification of 1,500 to locate areas of interest and at higher magnifications to detect the virus particles.

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1 Associated Electrical Industries Ltd., Harlow, Essex.

2 Hopkin & Williams Ltd., Chadwell Health, Essex, England.

3 Martin Christ, 336 Osterode/Herz, W. Germany.

Photographs were taken on Ilford special lantern contrasty plates ($3\frac{1}{4}$" x $3\frac{1}{4}$").

SEROLOGICAL TESTS

IMMUNOFLUORESCENCE

Both direct and indirect tests were used.

In the direct test, hyperimmune pig anti-Aujeszky's disease serum was conjugated with fluorescein-isothiocyanate (F.I.T.C.)[1] according to the method of Fraser and Haire (1969). The procedure consisted of conjugating whole serum with a celite-F.I.T.C.[2] mixture having 35 milligrams of the fluorochrome per ml. of serum, for 3 to 5 minutes in an icebath and then removing the celite granules by centrifugation at 4°C. The supernatant conjugate was then mixed with 4 times its volume of cold, 50 per cent saturated solution of ammonium sulphate. Precipitation occurred at 4°C in the course of 30 minutes and the precipitate was recovered by centrifugation at 3,000 r.p.m. for 30 minutes at 4°C. The deposited globulin was washed twice with 40 per cent saturated ammonium sulphate solution and then reconstituted in distilled water to half the original serum volume.

The conjugated globulin was passed through a Sephadex G-75 column using 0.01M P.B.S. to remove any unbound fluorochrome. The recovered globulin conjugate

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1 B.D.H. Chemicals Ltd., Poole.

2 Hopkin and Williams Ltd., Essex.

was concentrated to its original volume, if necessary, by dialysis against carbowax.

The conjugate was absorbed twice with uninfected PK15 cells at 37°C for 2 hours and the absorbent spun off at 10,000 r.p.m. for 30 minutes. The supernatant globulin was clarified through a millipore filter.

A commercial rabbit anti-pig globulin[1] was used in the indirect test.

Direct Test: Uninfected and infected coverslip cultures harvested at different periods post-infection were rinsed in Hank's B.S.S. prewarmed to 37°C and then fixed for 10 minutes in cold acetone and air-dried. The cultures were stained for 30 minutes by optimally diluted globulin conjugate in a humid atmosphere either at room temperature or 37°C. After washing for 10 to 15 minutes with 2 to 3 changes of P.B.S. (pH 7.2) the cultures were mounted in buffered glycerol[2].

Indirect Test: Acetone-fixed, air-dried infected and uninfected coverslip cultures were treated with undiluted pig anti-Aujeszky's disease serum for 30 minutes either at room temperature or 37°C. The cultures were washed for 10 to 15 minutes with 2 to 3

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1 Bacto F.A. Porcine Globulin Antiglobulin (Rabbit) Difco Laboratories, Detroit, Michigan, U.S.A.

2 Bacto F.A. Mounting fluid. Difco Laboratories, Detroit, Michigan, U.S.A.

changes of P.B.S. and then stained with rabbit anti-pig globulin conjugate for 30 minutes. The cultures were washed with frequent changes of P.B.S. for 10 to 15 minutes, mounted in buffered glycerol and examined.

COMPLEMENT-FIXATION TEST

The technique followed was that described by the W.H.O. Expert Committee on Respiratory virus Diseases (1959), the tests being carried out in W.H.O. plates.

Sera: Sera were inactivated at 56°C for 30 minutes after being diluted 1:4 in veronal-NaCl buffer (pH 7.2).

Diluent: Veronal-NaCl buffer containing 0.1 per cent bovine serum albumin was used.

Haemolytic System: A 3 per cent suspension of sheep erythrocytes in the diluent was sensitized with an equal volume of 5 minimum haemolytic doses (M.H.D.) of rabbit haemolysin[1] at 37°C for 10 minutes or at room temperature for 30 minutes.

Complement: Guinea pig serum obtained from Burroughs Wellcome Ltd. was used as haemolytic complement and titrations were carried out according to the manufacturer's instructions.

Antigens: CF antigens obtained by sonication of Aujeszky's disease virus-infected PK15 cells were used

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1 Burroughs Wellcome & Co., London.

for the detection of specific antibodies in rabbit anti-Aujeszky's disease sera. For titrating specific CF antigens in different cell cultures and tissues of chickens and rats experimentally infected with the virus, standard methods were followed using known positive and negative sera (Cruickshank, 1965).

Test: Serial two-fold dilutions of test sera in the diluent were carried out in 0.1 ml. volumes; the dilution ranged from 1:8 to 1:2048. Four units of specific CF antigens and 2 full haemolytic units of complement were added in 0.1 ml. volumes. Antigen control consisted of 4 units of specific antigens in 0.1 ml. amount to which were added 0.1 ml. of diluent and 0.1 ml. of complement. Serum controls contained the first three dilutions of test sera in 0.1 ml. volumes to which were added 0.1 ml. of diluent and 0.1 ml. of complement. The activity of the complement used in the test was titrated in triplicate to give 2, 1, $\frac{1}{2}$ and $\frac{1}{4}$ units in 0.1 ml. volumes. Diluent was then added in 0.2 ml. amounts.

In the tests designed to titrate the activity of different cell culture and tissue CF antigens, the antigen preparations were diluted in 2-fold series in duplicate in 0.1 ml. volumes. To one series of dilution was added a 1:50 dilution of a known positive

rabbit anti-Aujeszky's disease serum with a CF antibody titre of 1:1028 and to the other series was added a similar dilution of a healthy rabbit serum. Two full haemolytic units of complement were then added. When antigen preparations were anticomplementary, tests were replicated using 3, 4 and 5 full haemolytic units of complement.

The plates were held in the refrigerator for 18 to 20 hours and then incubated at 37°C for 20 to 25 minutes. A freshly prepared suspension of 3 per cent sheep erythrocytes optimally sensitized with rabbit haemolysin was added in 0.1 ml. amounts. The cell controls consisted of sensitized and unsensitized cells dispensed in 0.1 ml. amounts to which were added 0.3 ml. of diluent.

Plates were incubated at 37°C for 45 minutes, the contents being shaken every 15 minutes. Final readings were taken after the mixtures were allowed to settle in the refrigerator for 2-4 hours. The highest dilution showing 50 per cent haemolysis was taken as the end-point. Tests were replicated when necessary.

IMMUNODIFFUSION TEST

Standard Technique: A one per cent aqueous solution of Ion agar No. 2[1] containing thiomersal[2] to a final

1 Oxoid Division of Oxo Ltd., London.

2 The British Drug House Ltd., Poole.

concentration of 0.04 per cent was dispensed into plastic petri dishes in 4.5 ml. amounts to form a layer of approximately 2mm. thick. In some studies, circular wells, 5 mm. in diameter were cut in the agar with a standard punch so as to give a hexagonal pattern. The diffusion distance between wells was 2.5 mm. In other studies the wells were cut with a Feinberg Agar gel cutter[1] with a larger central well (12.5 mm. in diameter) and six small peripheral wells (4 mm. in diameter). The distance between the central and lateral wells was 6 mm.

Test: For the detection of specific ID antigens in Aujeszky's disease-infected cell cultures, the reference pig anti-Aujeszky's disease serum (5-10 times concentrated) was laid in the central well. The top and bottom wells were charged with normal pig serum or diluent. The test cultures were dispensed into the lateral wells. Similarly, for the detection of specific ID antibodies, a known positive cell culture ID antigen preparation was dispensed in the central well. Known positive and negative sera were laid in the top and bottom wells. The sera under examination were distributed in the lateral wells. Tests were carried out in triplicate. The plates were incubated in a

1 Shandon Scientific Co. Ltd., London.

humidified atmosphere at room temperature for periods up to 7 days. Precipitation lines induced by sera and antigens were regarded as being specific only when they formed a reaction of identity with known positive controls. Negative control antigens prepared from uninfected cell cultures and culture fluids on lines similar to those used for obtaining specific ID antigens, were also used to validate the positive reactions.

NEUTRALISATION TEST

Standard Technique: Sera were inactivated at 56°C for 30 minutes and then diluted in 2 or 4-fold series in maintenance medium. The dilutions were mixed in equal volumes with a virus suspension containing 1,000 $TCID_{50}$ per ml. The mixtures were first incubated at 37°C for 2 hours and then overnight in the refrigerator. To test the activity of the virus in the different serum-virus mixtures, 0.2 ml. of each mixture were then seeded into 4 to 5 tube cultures of PK15 cells containing 0.8 ml. of maintenance medium. The cultures were incubated at 37°C in a stationary position for 24 hours and then transferred to a roller-drum. The development of the CPE was visually assessed daily for a period of 5 days. The $TCID_{50}$ dose of the virus used in the test was also titrated under similar conditions. The 50 per cent end-point of virus neutralisation was

calculated by the method of Karber (1931) and the neutralising antibody titres were expressed as $\log_{10}$ units per ml.

ANALYSES

The data relating to experimental infections of mice, rats, chickens and cell cultures with Aujeszky's virus were subjected to statistical analyses by standard methods (Snedecor, 1957). In studies of parameters such as the effect of age of the experimental host or the dose of virus on clinical aspects of Aujeszky's infection, regression coefficients of the lines of the best fit were calculated by the method of least squares (Snedecor, 1957) and the significance of the values estimated by analysis of variance. Regressions were compared according to the method of Dawkins (1968). In the studies of the effect of different temperatures of incubation on Aujeszky's disease virus, half-life values were estimated according to the formula $t_{\frac{1}{2}} = \frac{0.3}{b}$ where 't' is the temperature of incubation and 'b' is the regression coefficient.

RESULTS

PATHOGENICITY FOR CELL CULTURES

PRIMARY CELL CULTURES

Most studies were carried out with the McFerran strain of Aujeszky's virus which was pathogenic for fowl embryo fibroblasts and kidney cells derived from chickens and five species of mammals (Table 5). In general, the cytopathic effects of the virus were manifest in about 18-24 hours. The earliest change recognised in unstained cultures was the emergence of small foci of refractile rounded cells in the cell sheet (Fig. 1). In stained coverslip cultures, the foci contained spherical shrunken cells with condensed cytoplasm and a central or eccentric, enlarged, hyperchromatic nucleus. Some of the adjacent cells also revealed loss of their characteristic polygonal shape and withdrawal of cytoplasmic processes together with enlargement and migration of the nucleoli. Disruption of the chromatin network and/or dissolution of the nuclear membrane also occurred but the cytoplasm showed no distinctive abnormalities other than slight granularity and increased acidophilia. Pari passu, the affected cells desquamated from the surface of the glass revealing multiple acellular foci in the cell sheet (Figs. 2 and 3).

At 24 hours or longer, most infected cultures showed several binucleate or trinucleate cells and also numerous

small collections of cells, the nuclei of which had joined together within syncytia. Whereas in unstained cultures the syncytia were recognised as granular refractile structures demarcated from the surrounding cell sheet, in stained cultures they appeared as clusters of nuclei without any separating cell boundaries.

Syncytia were rarely seen in infected fowl embryo and chicken kidney cell cultures, the predominant cytopathic change being rounding of affected cells(Figs. 4 and 5). When syncytia developed in chicken cell cultures they usually consisted of small clusters of nuclei with indistinct cell boundaries.

Nuclear inclusions were recognised only in the mammalian cell cultures. These inclusions were generally single, large and acidophilic or baso-acidophilic and occurred in isolated single cells as well as in the syncytia. Most inclusions were separated by a distinct halo from the nuclear membrane which was generally well-preserved and contained condensed nuclear matrix and chromatinic deposits, the hallmarks of the so-called Cowdry type A herpetic inclusion body.

In general, there were no large differences in the infectivity titres of the McFerran strain of virus for the different primary cell cultures, nor were there great differences in the amounts of virus produced in the different cell cultures (Table 5). However, the data

were based on a small number of titrations. Likewise, there were no differences in the onset, nature and magnitude of the CPE induced in primary cell cultures by the McFerran and Hungarian strains of virus. Preliminary observations also suggested that incubation of infected cultures on roller drums did not influence either the magnitude of the CPE or the infectivity titres.

Fowl embryo cells: The comparative susceptibilities of fowl embryo fibroblasts to Aujeszky's disease virus was investigated by titrating stock pools of the McFerran and Hungarian strains on fibroblast cultures prepared from small groups of fowl embryos ranging in age from 5 to 17 days. However, the results showed that there was no correlation between the age of the donor embryos and the infectivity titres of either virus ($F = 1.80$ and $F = 0.71$, d.f. 1, 4 respectively; Table 6). Likewise, when the same aliquots of the McFerran strain of virus were titrated on PK15 cells, no significant differences were detected between the mean infectivity titres for fowl embryo and PK15 cells ($t = 1.033$; $P > 0.20$, Table 13). Also there were no differences in the infectivity titres of the Hungarian strain of virus for the two types of cell cultures ($t = 0.327$; $P > 0.50$; Table 13). ~~Nevertheless, the mean infectivity titre of the McFerran strain~~

Chicken Kidney cells: Cell cultures prepared from the kidneys of chickens of 2 to 90 days of age supported the growth of Aujeszky's virus (Figs. 6 to 12; Table 7). Analysis of the data from this experiment suggested a negative correlation between the infectivity titres of the McFerran and Hungarian strains of virus and the age of chickens from which the renal cell cultures were obtained, but the values were not significant ($F = 5.72$; d.f. 1, 5 and $F = 3.07$, d.f. 1, 3 respectively). Similarly, no significant relationship was detected between the amounts of virus produced in the kidney cell cultures inoculated with the McFerran virus and the age of the donor chickens from which the cultures were derived ($F = 1.89$, d.f. 1, 4). Comparison of the data of mean infectivity and virus titres revealed no differences in the cytopathogenicity of the McFerran and Hungarian strains for chicken kidney cells ($t = 0.215$; $P > 0.50$ and $t = 0.279$; $P > 0.50$ respectively).

CELL LINES

The cytopathic effects induced by the McFerran strain of Aujeszky's virus on different established mammalian cell lines were similar to those observed on primary mammalian cell cultures (Table 8; Figs. 13-24). Small and large syncytia were regularly present in all of the infected cell lines and were easily recognised in unstained cultures at 24 to 48 hours post infection.

However, nuclear inclusions were indistinct or sparse in the infected LLC-MK2 and MDBK cultures. In general, there were no appreciable differences in the infectivity titres of the McFerran strain for primary cell cultures and cell lines. Likewise, no apparent differences existed in the amounts of virus produced in the primary and continuous cell cultures infected with this strain.

Effect of Age of Cells: The influence of the age of the PK15 cells on the onset of the CPE and infectivity titres of the McFerran strain of Aujeszky's virus was studied as follows:

Confluent monolayers were dispersed with a sodium versenate-trypsin mixture and approximately 1 x 10^5 cells/ml. were seeded into tubes with cover slips and allowed to monolayer at 37°C. Simultaneously aliquots of the cell suspension were added to each dilution of the virus for assay of infectivity. The final concentration of cells in this mixture was approximately 1 x 10^5 Cells/ml. The mixture was then shaken in a waterbath for 20 minutes at 37°C and dispensed in 1 ml. amounts in tubes containing coverslips and incubated at 37°C. Subsequently, batches of tube cultures were infected with different doses of the virus at days 1 to 6. Visual assessment of the CPE was confirmed by examination of stained coverslip cultures. It was observed that the mean day of the onset of the CPE was inversely related to

the age at which the cells were infected; the relationship being linear and significant (Table 9; Fig. 25). On the other hand, the age of the monolayers of PK15 cells had no influence on the infectivity titres of this strain of virus ($F = 0.0005$, d.f. 1, 5; Table 10).

Effect of Inoculum Size: The effect of the number of infective units of virus inoculated into cell cultures on the mean day of the induction of the CPE was investigated by infecting confluent day-old monolayer cultures of PK15 cells with different 10-fold serial dilutions of the McFerran and Hungarian strains of virus (Tables 11 and 12). It was noted that the time of appearance of the CPE was directly related to the amount of the input virus such that higher doses induced the CPE earlier than lower doses. In respect of each strain, the relationship between the dose of virus inoculated and the day of onset of the CPE was linear and significant ($F = 113.55^{**}$, d.f. 1, 181 and $F = 117.35^{**}$, d.f. 1, 60 respectively; Fig. 26). Furthermore, comparison of the two regressions of the mean day of onset of the CPE on the dose of the McFerran and Hungarian strains of virus revealed that the slopes of the regressions were identical ($F = 0.59$, d.f. 1, 241; Fig. 26) which suggested, that no strain differences existed in this regard.

Infectivity titres for PK15 cells : Strain differences: The Weybridge strain of Aujeszky's virus had a titre of $10^{4.10}$ $TCID_{50}$ per gram of brain tissue. On passage in PK15 cells the titres increased and the mean titres in the 2nd, 3rd and 4th passages were $10^{7.70}$, $10^{7.96}$ and $10^{7.57}$ $TCID_{50}$ per ml. respectively. Comparison of the mean infectivity titres of the McFerran, Hungarian and Weybridge strains for PK15 cells revealed that the cytopathogenicity of the McFerran strain was of a significantly higher order than that of either the Weybridge or the Hungarian strain (Table 14). Between the latter strains, there were no significant differences.

Comparison of the methods of harvesting the infected cell culture fluids for the production of virus pools and for the estimation of virus titres, showed that the supernatant fluids of the infected PK15 cells contained smaller amounts of virus than either frozen and thawed or sonicated cells in the culture fluids (Table 15) but the differences were not significant.

DEMONSTRATION OF VIRUS AND VIRAL ANTIGENS

Demonstration of Virus: Electron microscopic examination of cultures of PK15 cells injected with the McFerran strain of Aujeszky's virus confirmed the presence of particles having a morphology similar to those of the members of the herpesvirus group (Figs. 27 to 34).

Detection of Viral Antigens by Fluorescence: In general, there were no differences in the intensity of specific fluorescence in infected cultures stained by the direct and indirect methods.

In PK15 cells injected with a high dose of the McFerran strain ($10^{8.9}$ $TCID_{50}$ per ml.) no specific fluorescence was detected at 1, 2 and 3 hours post-infection. At 4 hours, small foci of fluorescent cells were recognised; individual cells revealed bright fluorescence of the nuclear membrane and small areas of brilliant fluorescence in the perinuclear zone usually on one side of the nuclear membrane (Figs. 35 and 36). In some cells, the perinuclear fluorescence was particulate whereas in others, the fluorescence appeared to have diffused towards the cell membrane. In some cells tiny foci of fluorescence were detected in the nuclear matrix (Fig. 36).

In cultures stained by haematoxylin-eosin, no distinctive abnormalities were recognised apart from some degree of cytoplasmic acidophilia, enlargement and hyperchromatism of the nuclei and swelling of the nucleoli. In occasional cells disintegration of the chromatin network was evident. In cultures stained with acridine-orange the only abnormalities apart from swelling of the nucleoli, and dispersion of the chromatin were granules of greenish to deep yellow fluorescence

which had developed in the nuclear matrix (Figs. 37 and 38).

At 6 hours, there were no further major changes although a greater number of cells showed specific fluorescence and cytoplasmic fluorescence was more prominent.

At 8 hours, there was further accumulation of fluorescent material in the cytoplasm and some cells showed large deposits of fluorescent antigens concentrated mostly in the perinuclear zone (Figs. 39 and 40). Changes in the morphology of the infected cells were more clearly recognised in cultures stained by acridine-orange or haematoxylin-eosin. In the acridine-orange preparations the nuclei of the infected cells showed a spectacular increase of greenish to deep yellow fluorescence (Figs. 41 and 42). In haematoxylin-eosin stained cultures, foci of rounded cells had developed and the CPE showed withdrawal of the cytoplasmic processes, condensation of the cytoplasma and nuclear anomalies ranging from intense hyperchromatism to pyknosis, fragmentation of the chromatin and the development of distinct Cowdry A type nuclear inclusions. Binucleate and trinucleate cells and syncytia were also seen (Figs. 43 and 44).

At 12-16 hours, degenerative changes had occurred in the nuclei and cytoplasm of the infected cells and the

nuclear inclusions were more prominent (Fig. 45). There was also an abundance of fluorescent material in the syncytia as well as in groups of individual cells (Fig. 40) throughout the expanse of the monolayer. In acridine-orange stained cultures, DNA fluorescence was still prominent within the syncytia as well as in individual infected cells.

Similar changes were observed in RK13 cells infected with the McFerran strain of virus. However, it was a feature of this cell line that Aujeszky's disease virus invariably produced numerous large multinucleate cells and that the nuclei contained an abundance of DNA fluorescence when stained with acridine-orange (Fig. 46).

There was also a close correlation between the infectivity titres, the development of viral antigens and the characteristic cytomorphological changes in the infected cultures (Table 16).

CF Antigens: Crude cell culture fluids and cells harvested from Aujeszky's virus-infected cultures contained no detectable amounts of CF antigens when tested against rabbit anti-Aujeszky's disease sera; but the antigens were demonstrable when the infected culture fluids were concentrated approximately 50 times by forced dialysis against carbowax or were precipitated with saturated ammonium sulphate solution and the precipitate reconstituted in a volume of diluent approx-

imately one fiftieth of that of the crude suspension (Table 17). Cell disruption procedures such as sonic disintegration or alternate freezing and thawing of infected cells facilitated the release of intracellular CF antigens but there were no significant differences in the relative titres of CF antigens obtained by concentration of infected cell culture fluids by physical methods or those harvested from infected cells by cell disruption procedures ($F = 0.42$, d.f. 2, 14; Table 17). Neither were there significant differences in the yields of CF antigens derived from infected RK13 or PK15 cell cultures ($t = 1.864$; $P > 0.05$).

Aujeszky's virus-specific CF antigens obtained from infected cell cultures were satisfactory for the detection of antibodies related to Aujeszky's virus in the sequential samples of sera from a rabbit that had been inoculated with 2 doses of heat-inactivated virus. The CF antibody titres in the rabbit sera were significantly higher than the neutralising antibody titres ($t = 3.732^{**}$; $P < 0.01$, Table 18). Specific CF antigens derived from injected RK13, PK15 or fowl embryo cell cultures had similar sensitivities. When a specimen of rabbit anti-Aujeszky's disease serum that had a neutralising antibody titre of 3.4 $\log_{10}$ units per ml. was titrated against CF antigens derived from RK13, PK15 and fowl embryo cell cultures the CF antibody titres

were 3.7, 3.7 and 3.6 $\log_{10}$ units per ml respectively, the values being the mean readings of three titrations. Likewise, the CF antigens obtained from fowl embryo cell cultures infected with the Hungarian or the McFerran strain of virus showed similar reactivities when tested against rabbit anti-Aujeszky's disease serum.

ID antigens: Aujeszky's virus-infected cultures concentrated by physical methods also contained antigens that reacted specifically in agar gel with pig anti-Aujeszky's hyperimmune serum and produced lines of precipitation (Figs. 47 and 48). In most cultures, there was a close correlation between the titres of specific immunodiffusion and complement-fixing antigens, the latter values always being the higher (Table 19). However, no relationship was evident between ID antigens and infectivity titres. For example, a batch of infected RK13 cell culture that had an infectivity titre of 6.5 $\log_{10}$ units per ml had an ID activity of the order of 1.6 $\log_{10}$ units per ml whereas, another batch of infected RK13 culture with a higher infectivity titre, namely $10^{7.0}$ units per ml had no demonstrable ID antigens.

As observed with CF antigens, the method of extraction of ID antigens from Aujeszky's virus-infected cultures did not influence the degree of the ID antigen activity. However, sonication of infected cells alone

did not engender reproducible titres of ID antigens (Table 19).

Precipitation reactions: Precipitation reactions between pig anti-Aujeszky's disease hyperimmune serum and ID antigens derived from virus-infected cell cultures occurred at 4°, 22° and 37°C but the rate of reaction was faster at 37° than at 22° or 4°C; the reaction proceeded at approximately the same speed at the latter temperature. For instance, when a culture that had an ID antigen titre of 32 units per 0.1 ml. was diffused in serial 2-fold dilutions against the reference serum and replicate plates were incubated at 4°, 22° or 37°C, precipitation lines were first detected at 6 hours in the plates incubated at 37°C. The titres at 8 and 24 hours were 1:8 and 1:32 respectively; in other words, the reaction was completed at 24 hours. On the other hand, in the plates held at 4° or 22°C, precipitation titres were first detected at about 18 hours and the reaction was completed on the 2nd or 3rd day.

Innumodiffusion antigens derived from Aujeszky's virus-infected cultures differed in their range of reaction with the specific antiserum. Of 11 preparations tested, 4 produced a single line of precipitation, 6 showed two lines and 1 gave three lines of precipitation when diffused against the reference serum (Fig. 54). In

immunodiffusion reactions characterised by the appearance of more than one precipitation line, the first line generally emerged after 18 hours of incubation at 4°, 22° and 37°C. Additional lines were recognised earlier when the plates were incubated at 37°C rather than at 4° and 22°C. However, continued incubation of the plates at 37°C often resulted in the merging, fading or complete extinction of one or more of the lines. The separation and preservation of the additional lines was achieved by prolonged incubation at 4°C. On occasions, when plates charged with the ID antigens and reference serum were incubated at 4°C, no more than a single line of precipitation developed until the 3rd or 4th day (Fig. 49), but when the plates were further incubated at 37°C an additional line appeared after about 24 hours (Fig. 50). In general, the precipitation lines tended to occur closer to the antibody wells than to the antigen wells.

The speed and range of reaction induced by specific ID antigens of Aujeszky's virus were not related to the host cells from which they were derived (Figs. 51 and 52). The results of limited studies also suggested that the concentration of agar in the gel had little influence on the range of the reaction; concentrations of 0.8 to 2.0 per cent were used and the reaction were similar.

Immunodiffusion reactions between cell culture specific ID antigens and reference pig or rabbit anti-Aujeszky's disease sera occurred only after these sera were concentrated 5 to 10 times (Fig. 53). But concentrated chicken sera did not produce precipitation lines. Some chicken sera gave non-specific precipitation lines when diffused against concentrated uninfected cell culture fluids; and the reactions were regarded as being non-specific (Fig. 54).

Preparations containing specific CF and ID antigens were active after exposure to temperatures ranging from 60° to 75°C for 20 minutes. The antigens were stable for several weeks when stored in the refrigerator.

VIRUS NEUTRALISATION TEST PARAMETERS

Thermal Inactivation: Recovery of Aujeszky's virus from the tissues of infected experimental hosts or cell cultures was occasionally confirmed by specific virus neutralisation tests using pig anti-Aujeszky's disease hyperimmune sera. Since in vitro neutralisation of most animal viruses by antibody is known to be considerably influenced by the time and temperature of incubation of the virus-serum mixtures, it was proposed to conduct a preliminary study of the kinetics of thermal inactivation of the McFerran strain of Aujeszky's virus employing temperatures that are commonly used in virus neutralisation tests.

From the results of the study it was evident that the rate of inactivation of the McFerran strain was directly related to the temperature of incubation and that the speed of inactivation was faster at 37° than at 22° or 4°C (Table 20). At each temperature of incubation, the rate of thermal inactivation of the virus was directly related to the time of exposure, the relationship being linear and highly significant; $\hat{Y}$ 4°C= 7.55 - 0.003.X; r = -0.921**; $\hat{Y}$ 22°C = 7.90 - 0.02.X; r = -0.998** and $\hat{Y}$ 37°C = 7.77 - 0.05.X; r = 1.00**, where $\hat{Y}$ is the regression of virus titre on time, X is the time of exposure and r, the correlation coefficient. The estimated half-life of the virus at 37°, 22° and 4°C was 5.45, 12.5 and 88.25 hours respectively. The results also suggested that incubation of virus with serum dilutions for 1 to 2 hours at 37°C or at room temperature or for 18 hours at 4°C is unlikely to cause significant denaturation of the input virus.

Effect of Time of Incubation: In a limited study, equal amounts of 8-fold serial dilutions of 3 samples of anti-Aujeszky's disease sera were incubated with virus containing 100 $TCID_{50}$ doses per 0.1 ml, for 1 or 2 hours at 37°C or for 2 hours at 37°C followed by overnight incubation in the refrigerator. The neutralising antibody titres appeared to be related to the length of

the incubation of the serum-virus mixtures (Table 21). For instance, sera that were first incubated at 37°C for 2 hours and then at 4°C for 18 hours had higher antibody titres than sera that were incubated with the virus at 37°C for 2 hours only, but the differences were not significant ($t = 0.917$; $P > 0.50$).

Effect of Dose: The influence of the dose of virus on the neutralising antibody titres was studied by incubating 10-fold serial dilutions of virus with 2-fold serial dilutions of pig anti-Aujeszky's disease sera at 37°C for two hours and then testing the virus-serum mixtures for residual virus by the inoculation of PK15 cell cultures. It was observed (Table 22) that the antibody titres were inversely related to the dose of virus, the relationship being linear and significant, such that with the increase of each 0.5 $\log_{10}$ unit of virus there was depression of antibody titres by 0.23 $\log_{10}$ units ($\hat{Y} = 3.92 - 0.47.X$; $r = -0.916^{**}$ where $\hat{Y}$ is the regression of antibody titre on dose, X is log virus dose and r is the correlation coefficient).

Similarly, the mean neutralising antibody titres in chicken anti-Aujeszky's disease sera estimated by titrating with 10^1 and 10^2 $TCID_{50}$ doses of virus were 2.60 ± 0.19 and 2.14 ± 0.18 respectively, but the difference was not significant ($t = 1.781$; $P > 0.05$; Table 22).

Effect of Complement on Neutralising antibody titres:
In a preliminary study, sequential samples of heat-inactivated rabbit anti-Aujeszky's disease sera were titrated for neutralising antibodies to Aujeszky's virus in the presence of 10 units of guinea pig complement and without added complement; 100 $TCID_{50}$ doses of virus were used in the test and the virus-serum mixtures were incubated at 37°C for two hours before seeding into PK15 cells. The addition of guinea pig complement enhanced the neutralising antibody titres and the differences in the values between the treated and untreated sera ranged from 0.4 to 2 $\log_{10}$ units (Table 23). Comparison of the differences in the virus-neutralising efficiency of the "early" and "late" sera revealed that the potency of "early" sera to neutralise the virus in the presence of complement was significantly higher than that of "late" sera in the presence of complement ($t = 16.87^{**}$, $P<0.01$ and $t = 2.545^{*}$, $P<0.05$ respectively). Sera collected on days 7 to 21 were designated as "early" sera and the samples obtained in the later periods were described as "late" sera.

From the results of another experiment, it was evident that the virus neutralising efficiency of rabbit anti-Aujeszky's disease sera was directly and significantly related to the amount of added guinea pig complement (Table 24), the line of best fit being linear

($\hat{Y} = 2.64 + 0.14.X$; $r = 0.702^{**}$ where $\hat{Y}$ is the regression of the antibody titre on the units of complement, X is the number of units of complement and r, the correlation coefficient). However, the addition of more than 5 to 10 units of complement had no further salutory effect on the antibody titres. Analysis of the data revealed that no significant differences existed in antibody titres obtained by treating the serum-virus mixtures with 10 to 20 units of complement ($t = 0.663$, $P > 0.50$).

The results of a limited study showed that rabbit complement was superior to guinea pig complement in augmenting the virus-neutralising efficiency of rabbit anti-Aujeszky's disease sera. When two samples of the rabbit serum were tested in the presence of 10 units of rabbit complement, the virus neutralising antibody titres were 4.30 and 4.45 $\log_{10}$ units per ml respectively which were in contrast to the titres of 3.85 and 3.85 $\log_{10}$ units per ml obtained in the presence of guinea pig complement.

TABLE 5

CYTOPATHIC EFFECTS IN PRIMARY CELL CULTURES INDUCED BY THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

CPE	Calf Kidney	Puppy Kidney	Ferret Kidney	Lamb Kidney	Piglet Kidney	Fowl embryo cells	Chicken Kidney
Rounding of cells	+	+	+	+	+	+	+
Focal desquamation	+	+	+	+	+	+	+
Syncytia	+	+	+	+	+	±	±
Nuclear inclusions	+	±	±	±	+	-	-
Mean infectivity titres	$10^{-6.23}$	$10^{-7.60}$	$10^{-6.20}$	ND	$10^{-7.50}$	$10^{-8.00}$	$10^{-7.21}$
Virus titres	5.50	6.20	6.20	ND	6.40	7.20	6.52

+ = observed - = not observed ± = sometimes observed ND = not done

TABLE 6

EFFECT OF AGE OF FOWL EMBRYOS FROM WHICH FIBROBLASTS WERE CULTURED ON THE REPLICATION OF THE MCFERRAN AND HUNGARIAN STRAINS OF AUJESZKY'S VIRUS

Age (days)	Infectivity titres ($_{10}TC1D_{50}$/ml.)	
	McFerran strain	Hungarian strain
5	$10^{-7.66}$	$10^{-7.25}$
7	$10^{-7.83}$	$10^{-7.68}$
9	$10^{-8.38}$	$10^{-7.50}$
11	$10^{-7.83}$	$10^{-7.40}$
13	$10^{-8.14}$	$10^{-7.50}$
17	$10^{-8.17}$	$10^{-7.17}$
Mean infectivity titres ± S.E.	$10^{-8.00} \pm 0.14$	$10^{-7.41} \pm 0.07$

TABLE 7

THE INFLUENCE OF THE AGE OF THE CHICKENS FROM WHICH KIDNEY CELLS WERE CULTURED ON THE REPLICATION OF THE MCFERRAN AND HUNGARIAN STRAINS OF AUJESZKY'S VIRUS

Age (days)	Infectivity titres ($\log_{10}TC1D_{50}$/ml.)		Virus titres ($\log_{10}TC1D_{50}$/ml.)	
	McFerran strain	Hungarian strain	McFerran strain	Hungarian strain
2	$10^{-8.17}$	$10^{-8.00}$		
4	$10^{-6.80}$	$10^{-7.20}$		
5	ND	ND	6.20	
9	$10^{-7.50}$		5.20	
15	ND	ND	6.50	7.20
30	$10^{-7.50}$	$10^{-7.00}$		
35	$10^{-7.30}$	$10^{-7.20}$	6.50	6.00
42	$10^{-7.00}$	$10^{-7.00}$	3.00	3.50
90	$10^{-6.20}$		4.20	
Mean titres ± S.E.	$10^{-7.21\pm0.24}$	$10^{-7.28\pm0.19}$	5.27 ± 0.57	5.57 ± 1.10

ND = no titrations were carried out

TABLE 8

CYTOPATHIC CHANGES INDUCED IN CELL LINES BY THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

CPE	BHK21	IK	LLC-MK2	HeLa	MDBK	MDCK	PK15	RK13
Rounding of cells	+	+	+	+	+	+	+	+
Focal desquamation	+	+	+	+	+	+	+	+
Syncytia	+	+	+	+	+	+	+	+
Nuclear inclusions	±	+	±	±	±	±	+	+
Mean infectivity titres*	$10^{8.30}$	$10^{6.00}$	ND	ND	$10^{7.59}$	ND	$10^{7.85}$	$10^{8.33}$
Virus titres*	8.00	6.20	ND	ND	6.75	6.70	8.75	8.17

\+ = observed - = not observed

ND = not done

*Expressed as the $\log_{10} TC1D_{50}$ units per ml.

TABLE 9

INFLUENCE OF AGE OF PK15 CELLS ON THE DAY OF ONSET OF CYTOPATHIC EFFECTS INDUCED BY THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Age (days)	N	CPE					Mean ± S.E.
		1	2	3	4	5	
0	29	24	5				1.17 ± 0.07
1	26	15	10		1		1.35 ± 0.11
2	26	12	12	1	1		1.65 ± 0.15
3	28	9	13	6			1.90 ± 0.14
4	28	12	14	2			1.54 ± 0.12
5	26		19	6	1		2.31 ± 0.11
6	20	4	9	4	2	1	2.35 ± 0.24

N = number of cultures showing CPE

TABLE 10

THE INFLUENCE OF THE AGE OF CELLS ON THE INFECTIVITY TITRES IN PK15 CELLS INOCULATED WITH THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Age (days)	Titre ($\log_{10}$ TC1D$_{50}$/ml.)
0	$10^{-8.13}$
1	$10^{-7.88}$
2	$10^{-7.88}$
3	$10^{-8.00}$
4	$10^{-8.25}$
5	$10^{-7.80}$
6	$10^{-8.07}$

TABLE 11

INFLUENCE OF THE SIZE OF INOCULUM ON THE DAY OF ONSET OF CYTOPATHIC CHANGES IN PK15 CELLS INFECTED WITH THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Inoculum ($log_{10}TCID_{50}$/ml.)	N	CPE					Mean ± S.E.
		1	2	3	4	5	
3.2	48	37	11				1.23 ± 0.61
2.2	56	29	27				1.46 ± 0.07
1.2	55	10	32	11	2		2.09 ± 0.10
0.2	24	12	8	3	1		2.71 ± 0.18

N = number of cultures showing CPE

TABLE 12

MEAN DAYS OF ONSET OF THE CYTOPATHIC EFFECTS IN PK15 CELLS INFECTED WITH DIFFERENT DOSES OF THE HUNGARIAN STRAIN OF AUJESZKY'S VIRUS

Inoculum ($log_{10}TCID_{50}$/0.1 ml.)	N	CPE					Mean ± S.E.
		1	2	3	4	5	
6.7	8	8					1.00 ± 0.00
5.7	8	8					1.00 ± 0.00
4.7	12	10	1	1			1.25 ± 0.18
3.7	8	0	4	4			2.50 ± 0.17
2.7	8	0	3	3	2		2.88 ± 0.29
1.7	12	0	2	8	2		3.00 ± 0.17
0.7	6	0	0	2	2	2	4.00 ± 0.36

N = number of cultures showing CPE

TABLE 13

COMPARISON OF THE INFECTIVITY TITRES* OF THE MCFERRAN, HUNGARIAN AND WEYBRIDGE STRAINS OF AUJESZKY'S VIRUS FOR PK15 CELLS

	McFerran strain	Weybridge strain	Hungarian strain
	8.00	7.25	7.00
	8.09	7.50	7.39
	8.50	7.53	7.50
	8.50	7.70	7.58
	8.50	7.95	7.63
	8.63	8.08	
		8.20	
		8.20	
		8.20	
Mean ± S.E.	8.37 ± 0.37	7.84 ± 0.16	7.45 ± 0.09

*Expressed as the negative logarithm of the number of infective units per ml.

TABLE 14

SIGNIFICANCE OF THE DIFFERENCES IN THE MEAN INFECTIVITY TITRE OF THE MCFERRAN, THE HUNGARIAN AND THE WEYBRIDGE STRAINS OF AUJESZKY'S VIRUS FOR PK15 CELLS

Comparison	Difference	S.E. of difference	d.f.	t	P
The McFerran and the Hungarian strains	$10^{-0.92}$	0.346	10	2.659*	<0.025
The McFerran and the Weybridge strains	$10^{-.53}$	0.288	14	1.806	>0.05
The Hungarian and the Weybridge strains	$10^{-0.39}$	0.533	14	0.732	>0.40

TABLE 15

COMPARISON OF VIRUS TITRES* OBTAINED IN DIFFERENT HARVESTS FROM PK15 CELLS INFECTED WITH THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

	Supernatant fluid of infected cell culture	Frozen and thawed infected cells and culture fluid	Sonicated infected cells and culture fluid
	7.20	8.20	8.30
	7.70	7.40	7.40
	7.40	7.50	
	6.70	8.50	
	6.80	9.50	9.50
	5.80	6.90	7.00
	5.50	5.70	
	5.00	7.02	7.50
Mean ± S.E.	6.51 ± 0.34	7.59 ± 0.39	7.84 ± 0.79

*Expressed as the negative logarithm of the number of infective units per ml.

TABLE 16

CORRELATION BETWEEN INFECTIVITY TITRES AND RESULTS OF IMMUNOFLUORESCENT AND CYTOLOGICAL OBSERVATIONS IN PK15 CELLS INFECTED WITH $10^{8.9}TCID_{50}$ DOSES PER ML. OF THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Hours post-infection	Infectivity titres ($log_{10}TCID_{50}/ml.$) ± S.E.		Immuno-fluorescence	Acridine-orange staining	H and E staining
	Cell culture fluid	Infected cells and culture fluid			
2			-	-	-
4	4.67 ± 0.36	4.83 ± 0.48	+	±	±
8	6.33 ± 0.36	7.40 ± 0.31	++	++	++
12	7.50 ± 0.31	7.63 ± 0.35	+++	+++	+++
16	7.60 ± 0.36	8.20 ± 0.14	+++	+++	+++
20	8.25 ± 0.50	8.25 ± 0.50	+++	+++	+++
24	7.67 ± 0.48	9.25 ± 0.48	+++	+++	+++

+ to +++ = Degree of cytolomorphological changes, specific fluorescence and DNA staining.

\- = Absence of CPE, fluorescence and DNA staining.

± = Query CPE, fluorescence and DNA staining.

TABLE 17

DEMONSTRATION OF COMPLEMENT-FIXING ANTIGENS** IN AUJESZKY'S VIRUS-INFECTED CULTURES

Batch	Cell culture	Cells fluids concentrated (X50) by dialysis	Cell bound CF Antigens*	Released CF antigens	
				Concentrated (X50) by dialysis	Concentrated (X50) by treatment with $(NH_4)_2SO_4$
1	RK13	2.3	1.7	2.3	2.0
2		2.3	1.7	2.3	
3		2.0	2.6		
4		1.7			
5		3.5			
6	PK15		2.6	2.9	
7			2.6	2.6	2.6
8	Fowl embryo fibroblasts	3.5			

* Obtained by sonication of infected cells.

** Expressed as the negative logarithm of the number of CF units per ml.

TABLE 18

COMPARISON OF TITRES OF COMPLEMENT-FIXING AND NEUTRALISING ANTIBODIES IN RABBIT ANTI-AUJESZKY'S DISEASE SERUM

Days post-inoculation	CF antibodies*	N antibodies*
0	< 1.0	< 1.0
7	2.2	< 1.0
13	3.1	2.4
21	3.7	3.0
28	3.7	3.0
35	3.7	3.1
42	3.4	3.4
48	3.7	3.4
56	4.0	3.4
97	4.0	3.1
144	3.4	3.1

*Expressed as the negative logarithm of the number of antibody units per ml.

TABLE 19

RELATIONSHIP BETWEEN THE TITRES OF IMMUNO DIFFUSION AND COMPLEMENT-FIXING ANTIGENS AND INFECTIVITY TITRES IN AUJESZKY'S VIRUS-INFECTED CELL CULTURES

Batch	Cell culture	Extraction procedure	Titres		
			ID*	CF*	Infectivity ($log_{10}TC1D_{50}$/ml)
1	PK15	Sonication of infected cells	1.9	2.5	8.20
2	"	" " "	<1.0	2.6	8.00
3	"	Freezing and thawing of cultures and concentration (X50) by dialysis	1.9	2.6	6.20
4	"	" " "	1.9	2.6	7.78
5	"	Freezing and thawing of cultures and treatment with ammonium sulphate	1.6	2.2	8.00
6	"	" " "	1.6	2.2	7.80
7	RK13	Sonication of infected cells	1.9	2.5	8.77
8	"	" " "	1.6	2.2	7.20
9	"	Freezing and thawing of cultures and concentration (X50) by dialysis	1.6	2.9	6.50
10	"	Freezing and thawing of cultures and treatment with ammonium sulphate	<1.0	2.6	7.00
11	Fowl embryo cells	Sonication of infected cells	<1.0	1.9	
12	"	Freezing and thawing of cultures and concentration (X50) by dialysis	1.0	2.5	
13	"	" " "	1.6	2.9	7.70

. *Expressed as the negative logarithm of the number of units per ml.

TABLE 20

THERMAL INACTIVATION OF THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS*

AT DIFFERENT TEMPERATURES

Hours	Temperature (°C)		
	4	22	37
0	7.60 ± 0.23	7.80 ± 0.16	7.90 ± 0.16
2			7.70 ± 0.13
4		7.75 ± 0.16	7.40 ± 0.22
6	7.57 ± 0.25	7.75 ± 0.16	
8			
12	7.32 ± 0.12	7.63 ± 0.21	7.10 ± 0.18
18	7.38 ± 0.12		
24	7.38 ± 0.12	7.30 ± 0.20	
36	7.38 ± 0.12		
48	7.50 ± 0.17	6.75 ± 0.16	
72	7.37 ± 0.21		
96	7.25 ± 0.16		
120	7.12 ± 0.23		
160	7.12 ± 0.23		
210	6.94 ± 0.22		

*Expressed as the negative logarithm of the number of neutralising antibody units per ml ± standard error.

TABLE 21

THE EFFECT OF TIME OF INCUBATION OF THE SERUM-VIRUS MIXTURES ON NEUTRALISING ANTIBODY TITRES IN ANTI-AUJESZKY'S DISEASE SERA

Serum	Incubation period (hours at 37°C)		
	1	2	2 + overnight at 4°C
Pig	3.49	3.55	3.80
Rabbit		2.60	3.00
Chicken	3.26	3.38	3.83

TABLE 22

THE EFFECT OF DOSE OF VIRUS ON THE NEUTRALISING ANTIBODY TITRES IN PIG AND CHICKEN ANTI-AUJESZKY'S DISEASE SERA

Serum	Dose of virus ($log_{10}TCID_{50}$/0.1 ml.)							
	0.1	1	2	3	4	5	6	7
Pig	3.84	3.44	3.16	2.85	2.40	1.30	1.00	<1.00
		3.71	2.30	2.00				
		3.71		2.81	2.20	1.60	1.00	<1.00
	3.71	3.13	3.13	2.81	2.20	1.30	1.00	<1.00
Chicken			2.34	<1.90				
			2.89	2.28				
			2.40	<1.90				
		2.60	2.20					
		2.20	1.90					
		2.60	2.08					
		2.38	1.90					
		2.60	<1.90					
		2.15	<1.90					
		3.70	3.10					

TABLE 23

THE EFFECT OF ADDITION OF GUINEA PIG COMPLEMENT* TO THE SERUM-VIRUS MIXTURES ON THE NEUTRALISING ANTIBODY TITRES IN RABBIT ANTI-AUJESZKY'S DISEASE SERUM

Days post-inoculation	Without added C'	With added C'
0	< 1.0	< 1.0
7	< 1.0	1.2
10	1.4	3.4
13	2.4	3.5
16	3.1	3.7
21	3.0	3.7
28	3.0	4.0
48	3.4	3.4
97	3.1	3.4
144	3.1	3.5

*Added to a final concentration of 10 hundred per cent haemolytic units in the serum-virus mixtures

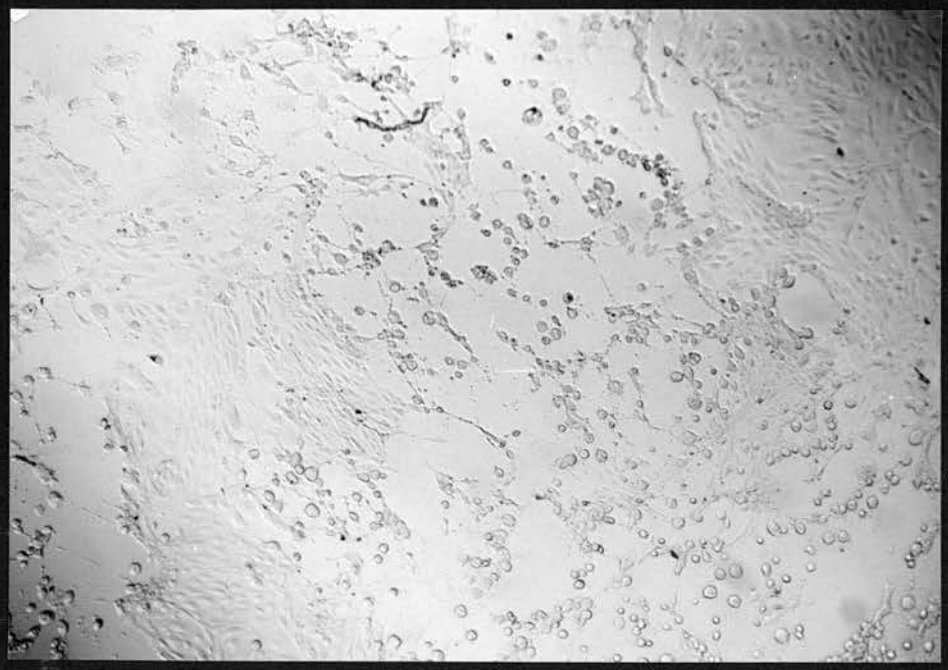
TABLE 24

THE INFLUENCE OF THE NUMBER OF UNITS OF GUINEA PIG COMPLEMENT (C') ADDED TO SERUM-VIRUS MIXTURES ON THE NEUTRALISING ANTIBODY TITRES IN RABBIT ANTI-AUJESZKY'S DISEASE SERUM

Units of C'	Serum			
	1	2	3	4
0	1.12	2.95	2.98	2.35
<0.3	1.90	3.40		
2.5	3.55	3.70		
5.0	3.55	3.70	3.70	3.40
10.0	3.85	3.85	4.00	3.40
20.0	3.55	3.40	4.00	

Fig. 1. - Cytopathic effects induced on primary bovine kidney cell culture 24 hours after infection with the McFerran strain of Aujeszky's virus. x 90.

Fig. 2. - Foci of desquamation of rounded cells in primary lamb kidney cell culture 24 hours after infection with the virus. x 90.



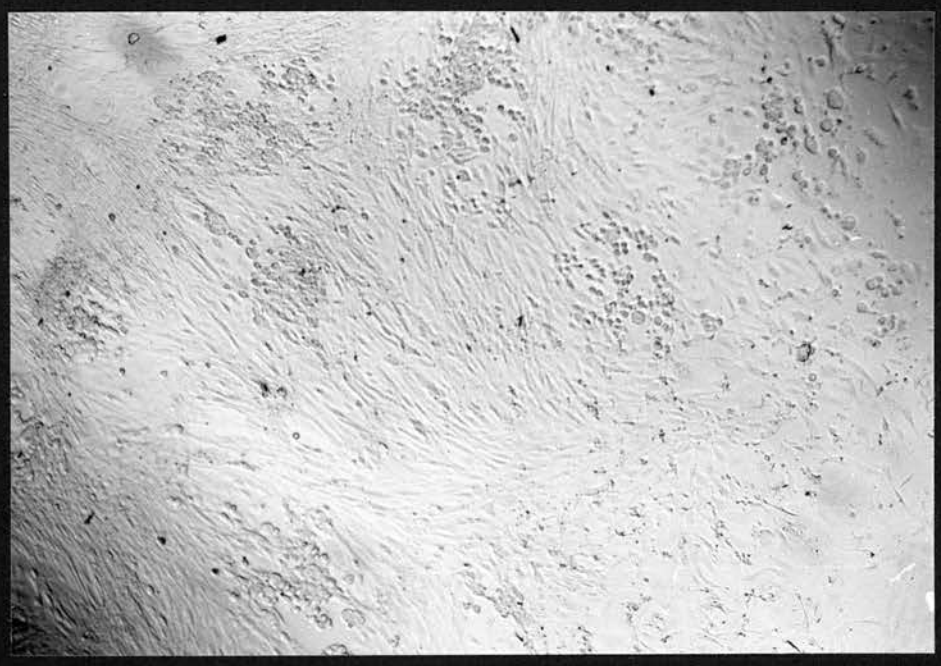


Fig. 3. - Massive desquamation of degenerated cells in primary pig kidney cell culture 24 hours after infection with the McFerran strain of Aujeszky's virus. x 90.

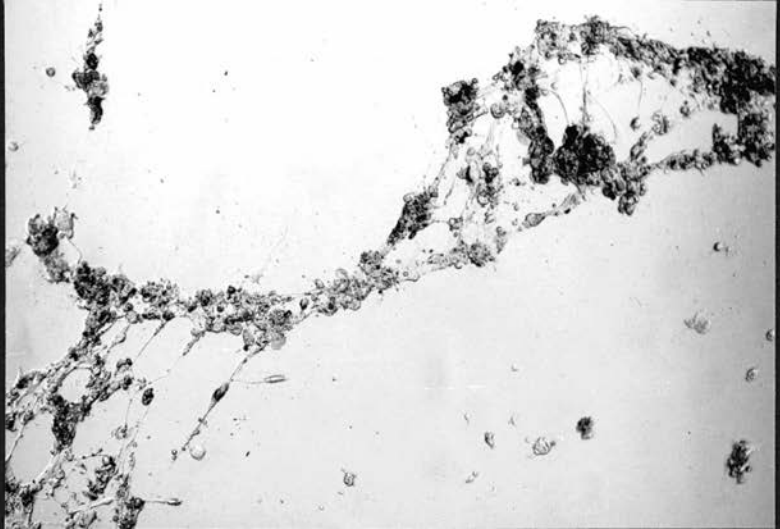
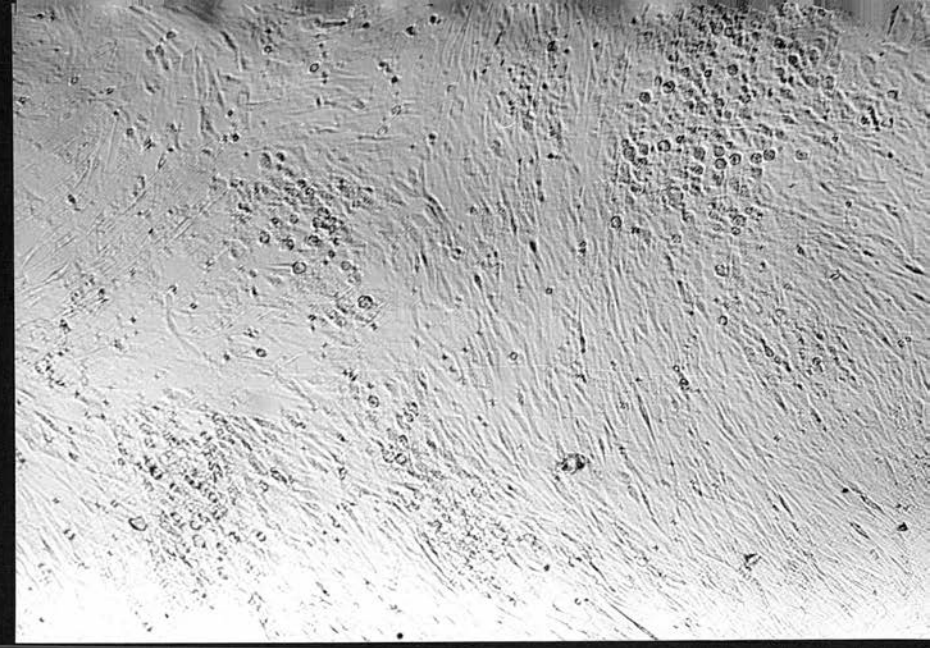
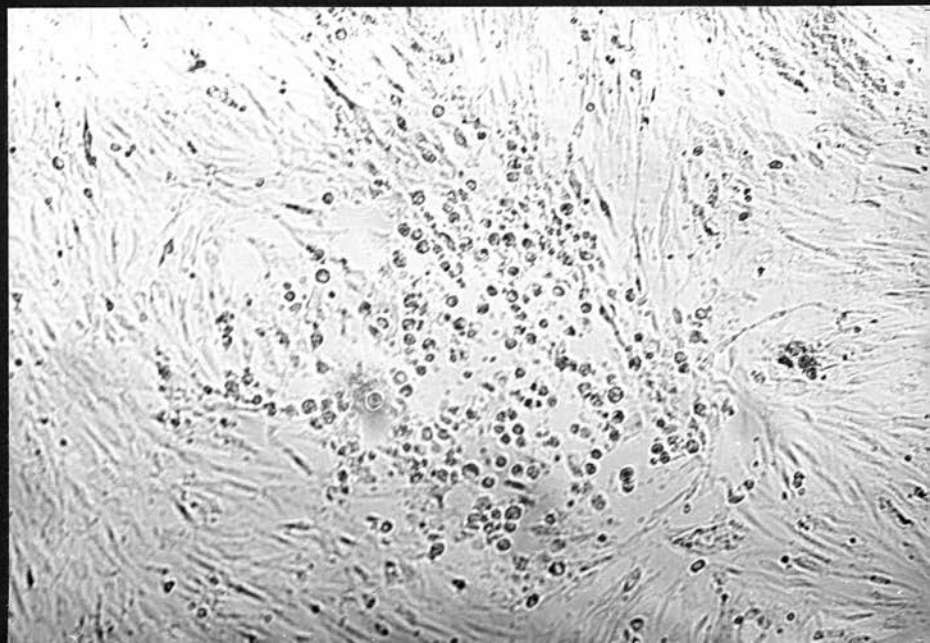


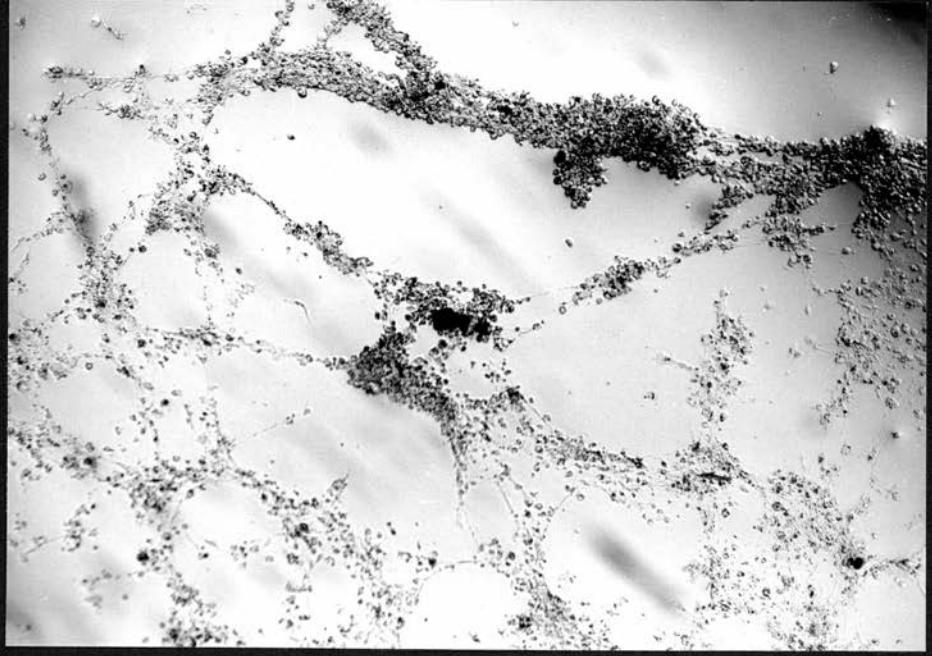
Fig. 4. - Commencement of rounding degeneration in fowl embryo fibroblast culture 48 hours after infection with 70 $TCID_{50}$ of the McFerran strain of Aujeszky's virus. x 90.

Fig. 5. - Another area in the same culture showing a focus of rounded and shrunken cells. x 90.





Figs. 6 and 7. - Development of clusters of degenerate rounded cells in primary renal cell cultures derived from 9 days old chickens 24 hours after infection with the McFerran strain of Aujeszky's virus. x 90.



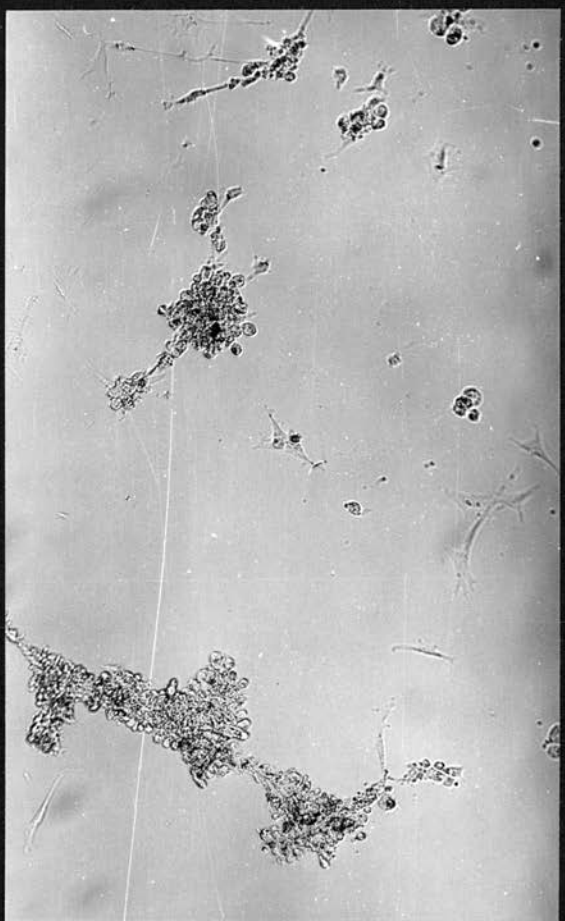
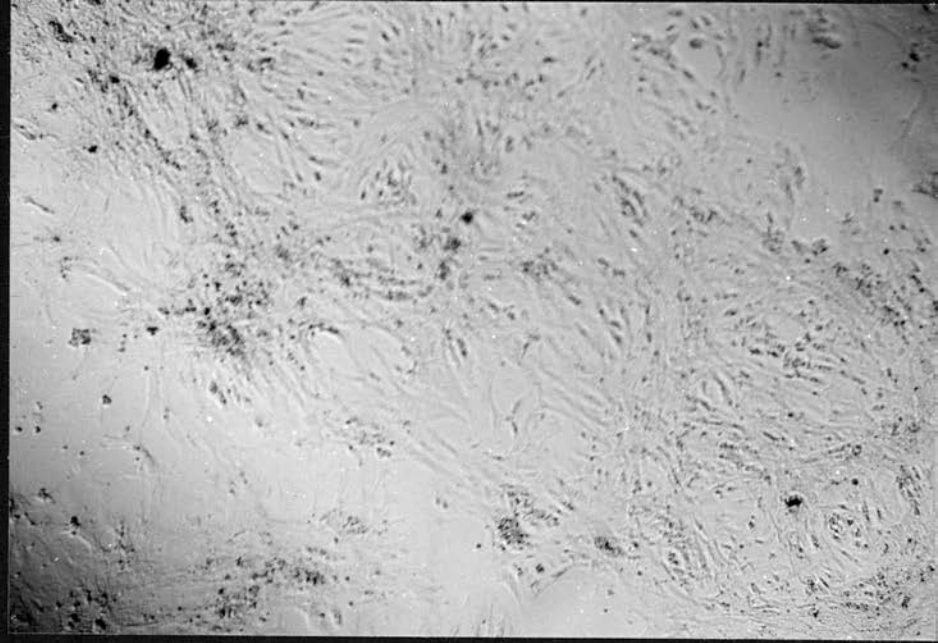


Fig. 8. - Cytopathic changes characterised by widespread necrosis of cells induced at 24 hours by the Hungarian strain of Aujeszky's virus in primary kidney cell cultures obtained from 28 days old chickens. x 90.

8

Fig. 9. - Uninfected 12 days' old monolayer culture of kidney cells prepared from 42 days old chickens. x 90.

Fig. 10. - Large foci of desquamation in 8 days old cultures 48 hours after infection with the McFerran strain of Aujeszky's virus. x 90.



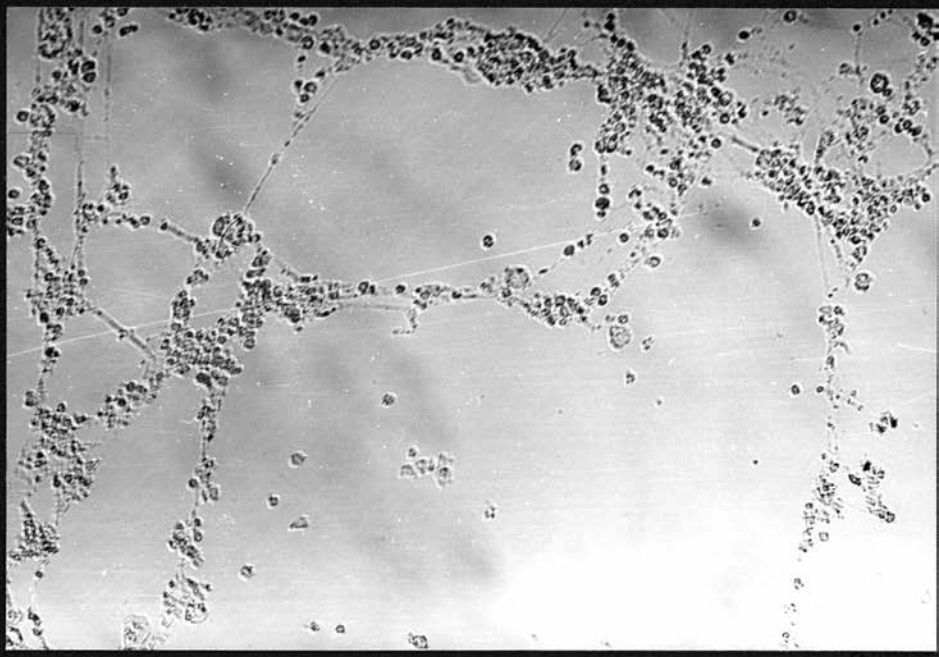


Fig. 11. - Uninfected kidney cell culture from 84 days old chickens. x 90.

Fig. 12. - CPE 72 hours after infection with 1000 $TC1D_{50}$ of the McFerran strain of Aujeszky's virus in which the rounded cells are connected by long, fibrils of condensed cytoplasm showing a beaded appearance in places. x 90.

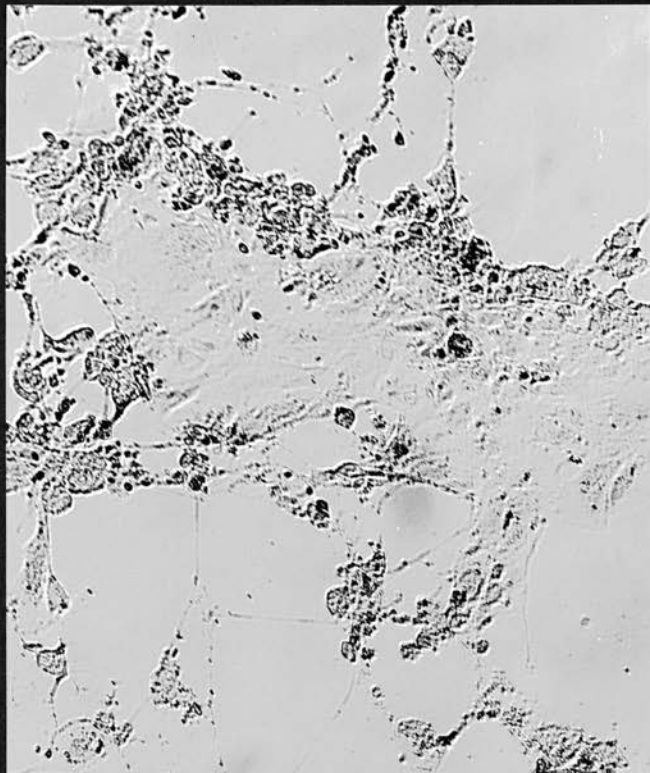
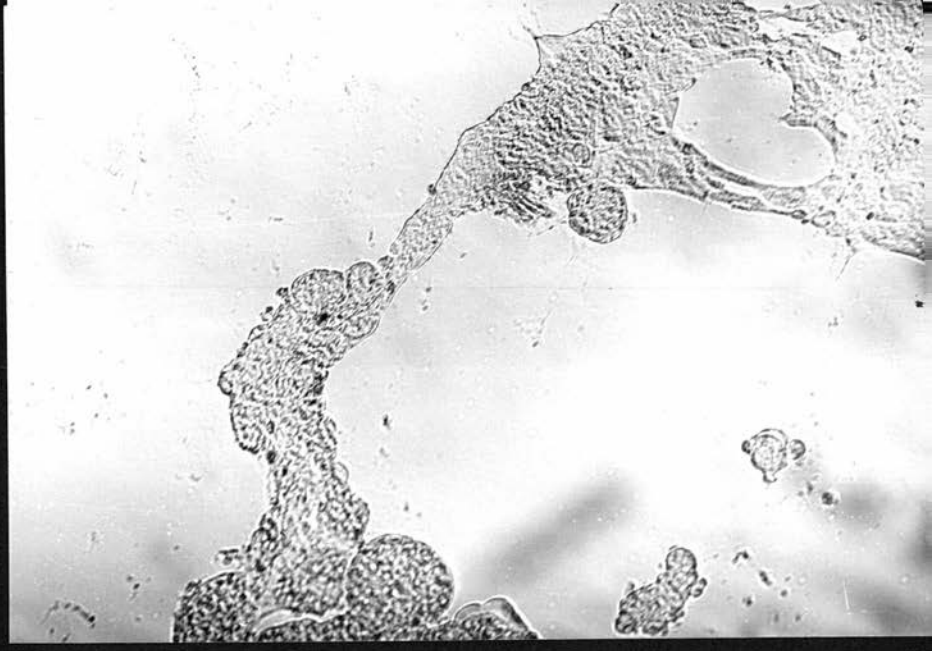


Fig. 13. - Syncytia in a pig kidney cell line 12 hours after infection with $10^{8.0}$ $TC1D_{50}$ of the McFerran strain of Aujeszky's virus. x 90.

Fig. 14. - Development of acellular foci in a pig kidney cell line 24 hours after infection with $10^{4.0}$ $TC1D_{50}$ of the McFerran strain of Aujeszky's virus. x 90.



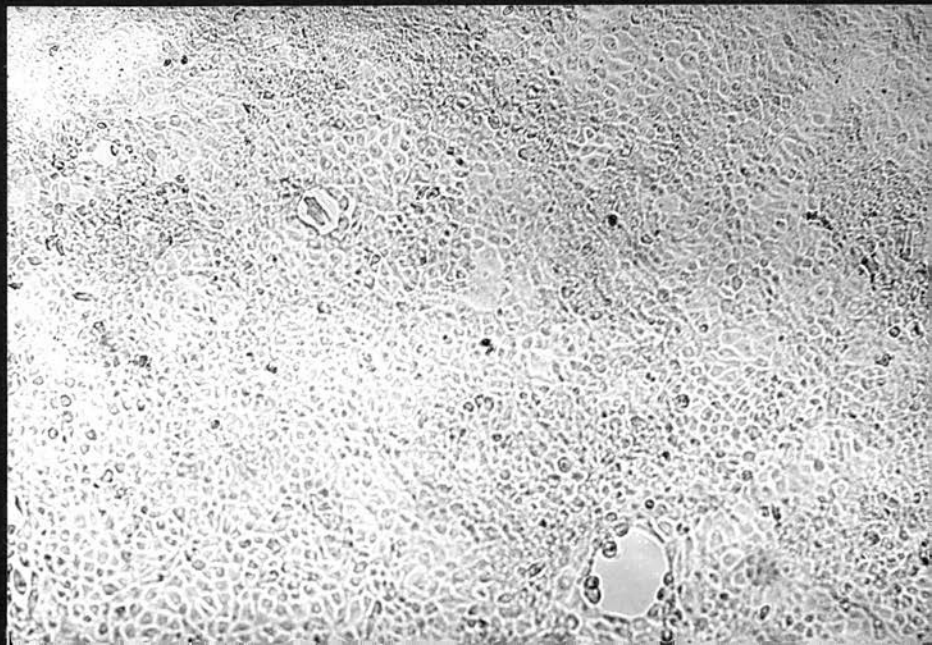
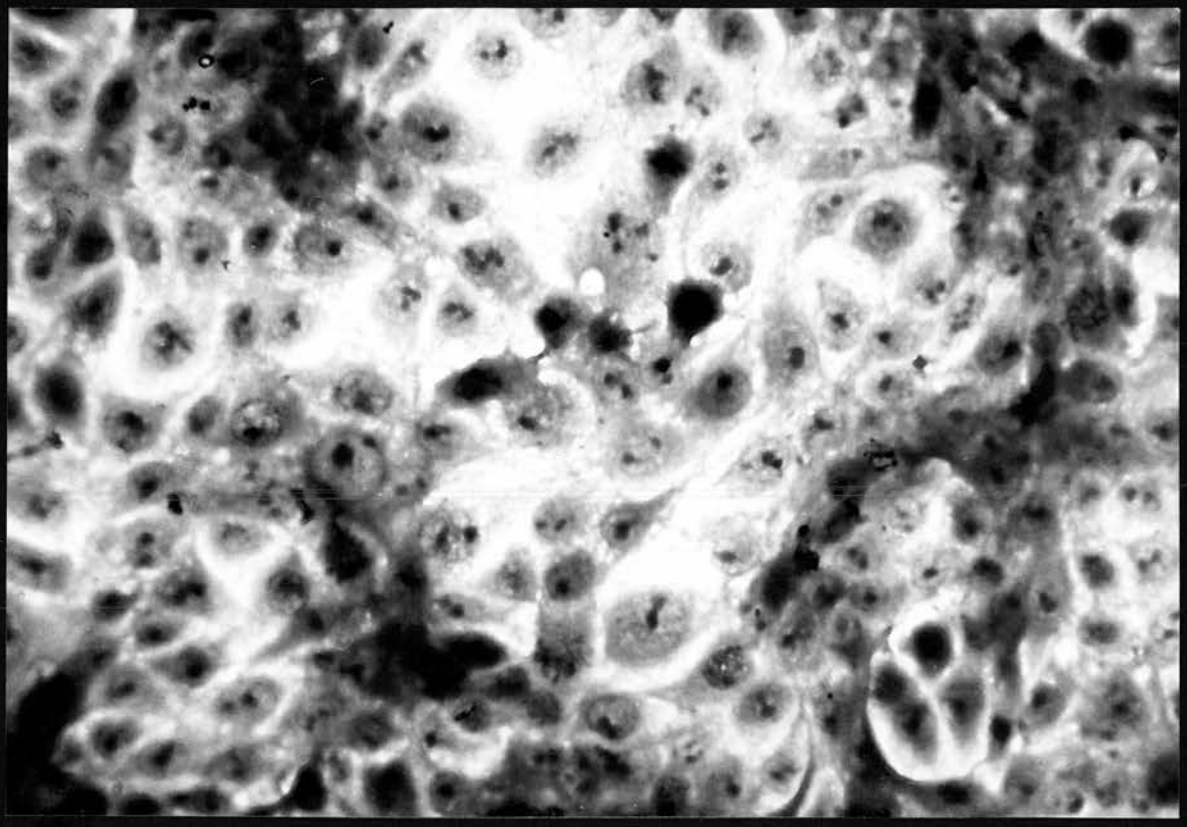


Fig. 15. - Development of large multiple syncytia connected by condensed strings of cytoplasmic processes in RK13 cell culture 22 hours after infection with a high dose of the McFerran strain of Aujeszky's virus. x 90.

Fig. 16. - Foci of desquamation in lamb kidney cell line 48 hours after infection with 100 $TC1D_{50}$ of the McFerran strain of Aujeszky's virus. The foci show piled up rounded cells. x 90.

Fig. 17. - Uninfected PK15 cells H & E. x 100.

Fig. 18. - Development of a microplaque in PK15 cells 48 hours after infection with 100 $TCID_{50}$ of the McFerran strain of Aujeszky's virus H & E. x 100.



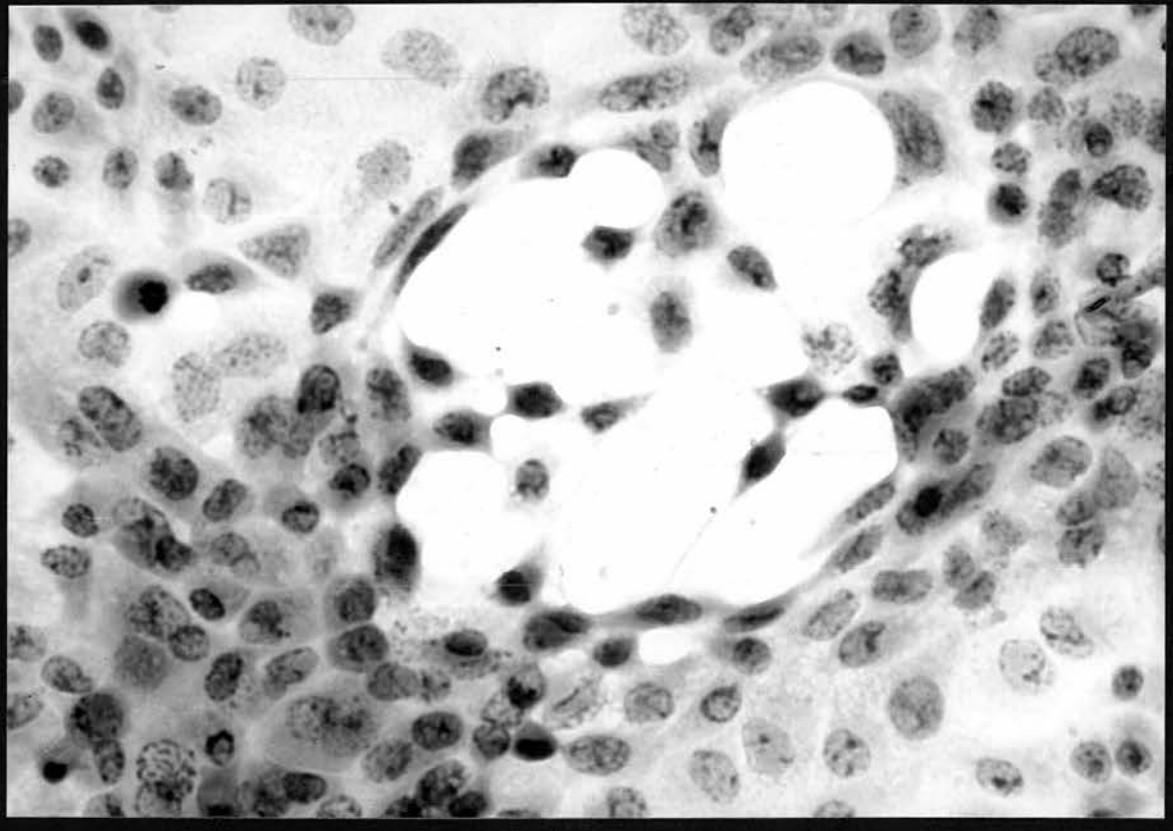
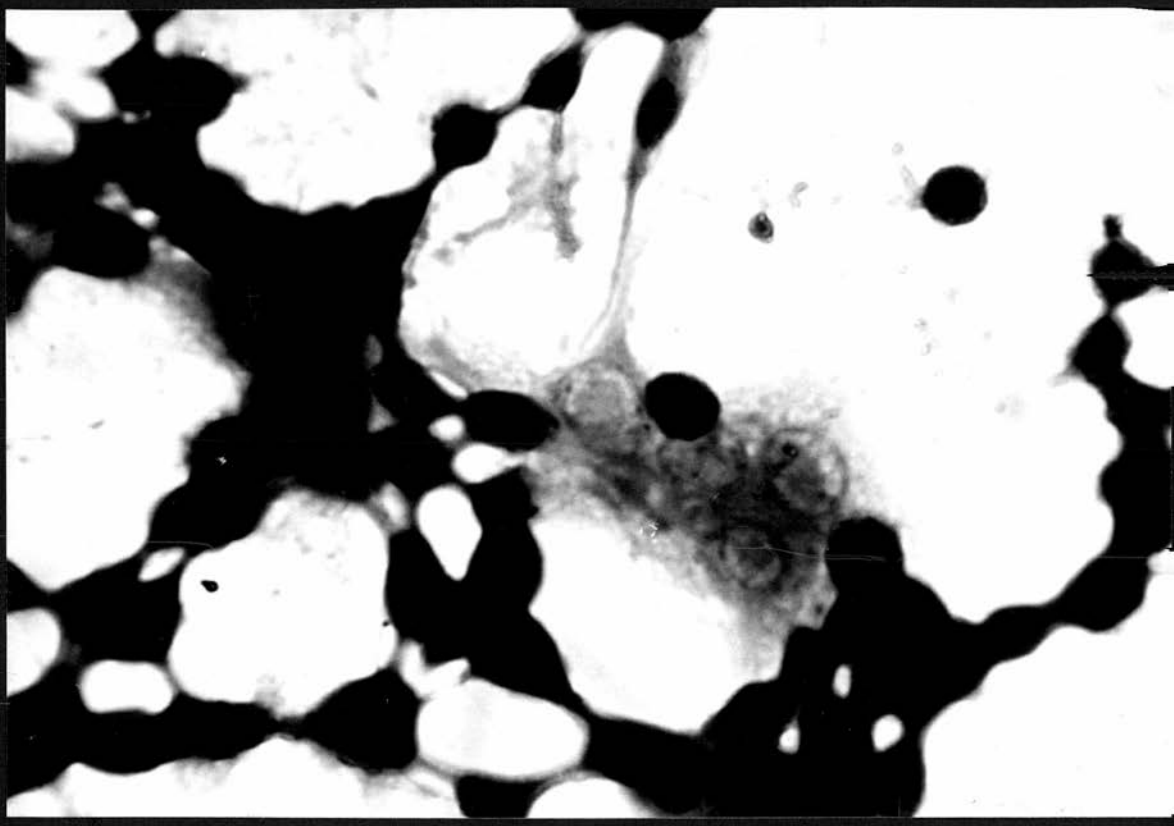
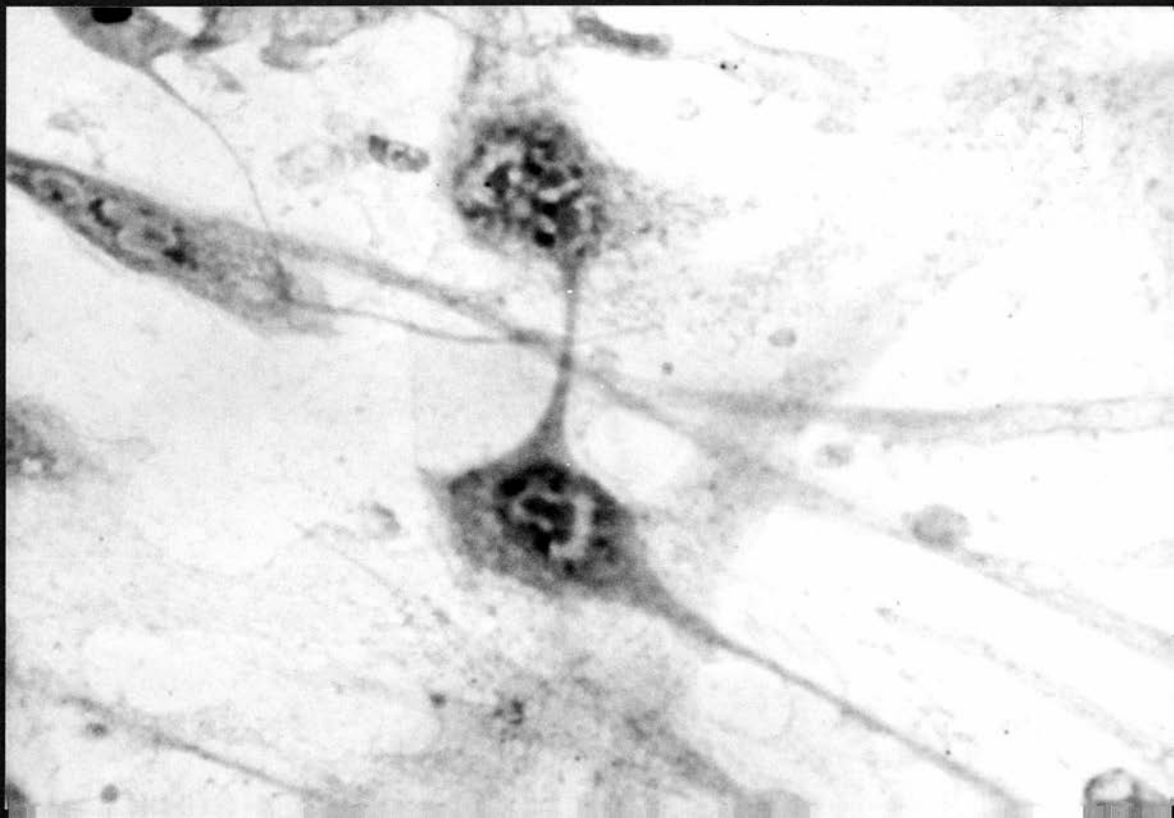


Fig. 19. - PK15 cell culture 9 hours after infection with a high dose of the McFerran strain of Aujeszky's virus showing clusters of intensely hyperchromatic cells in the vicinity of a small syncytium. The nuclei in the syncytium contain Cowdry A type inclusions H & E. x 200.

Fig. 20 - Isolated cells showing nuclear inclusion H & E. x 200.



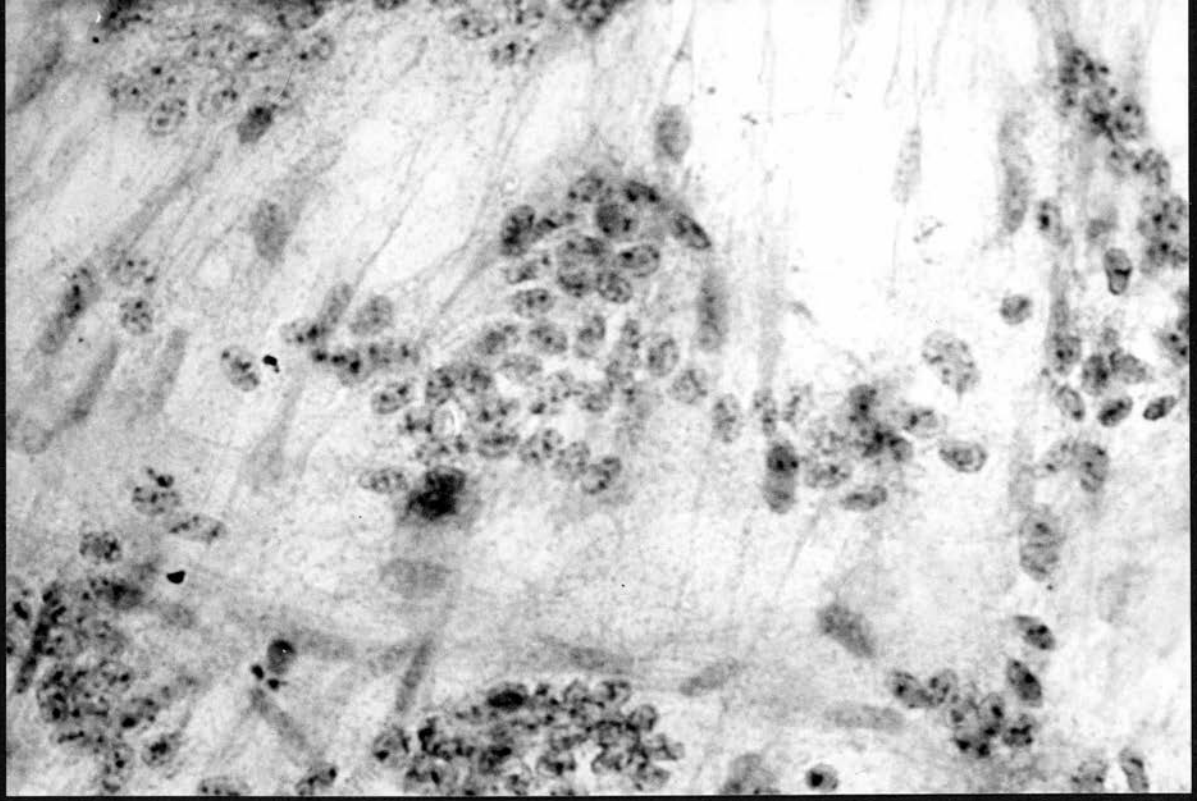
19.

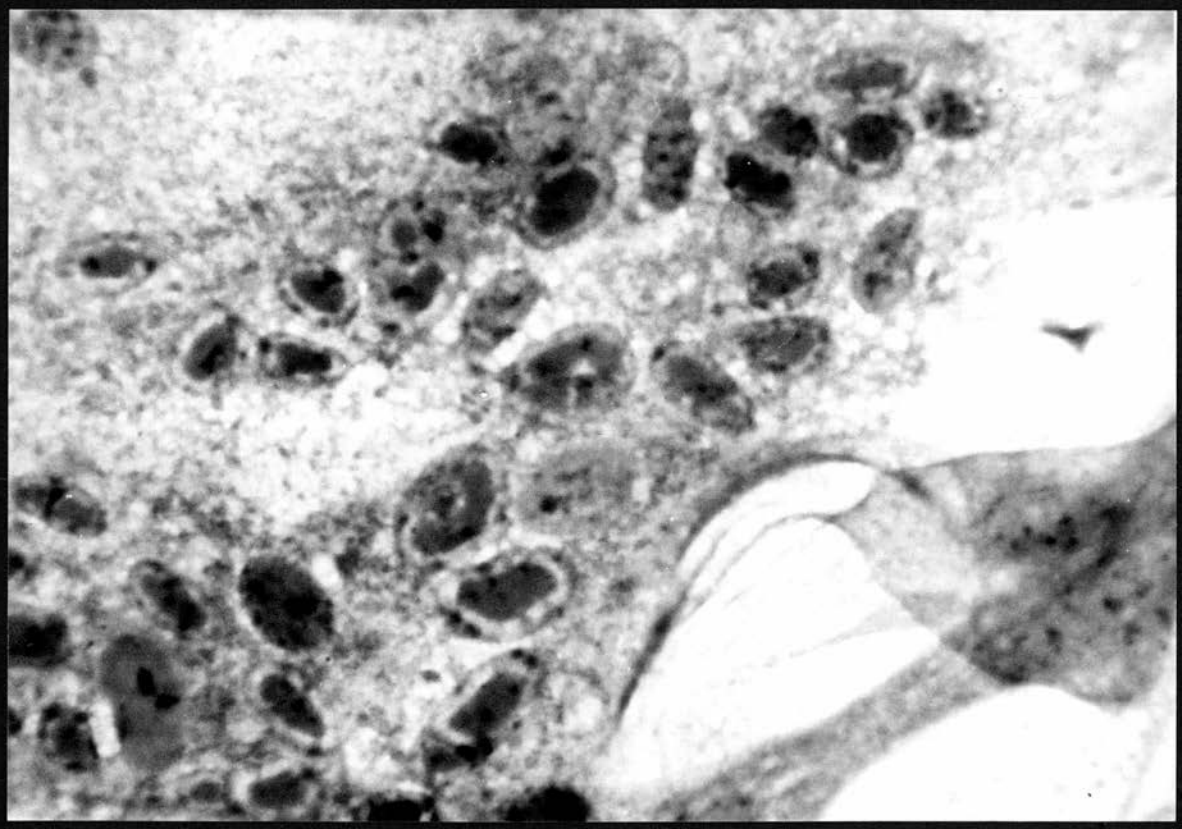


20

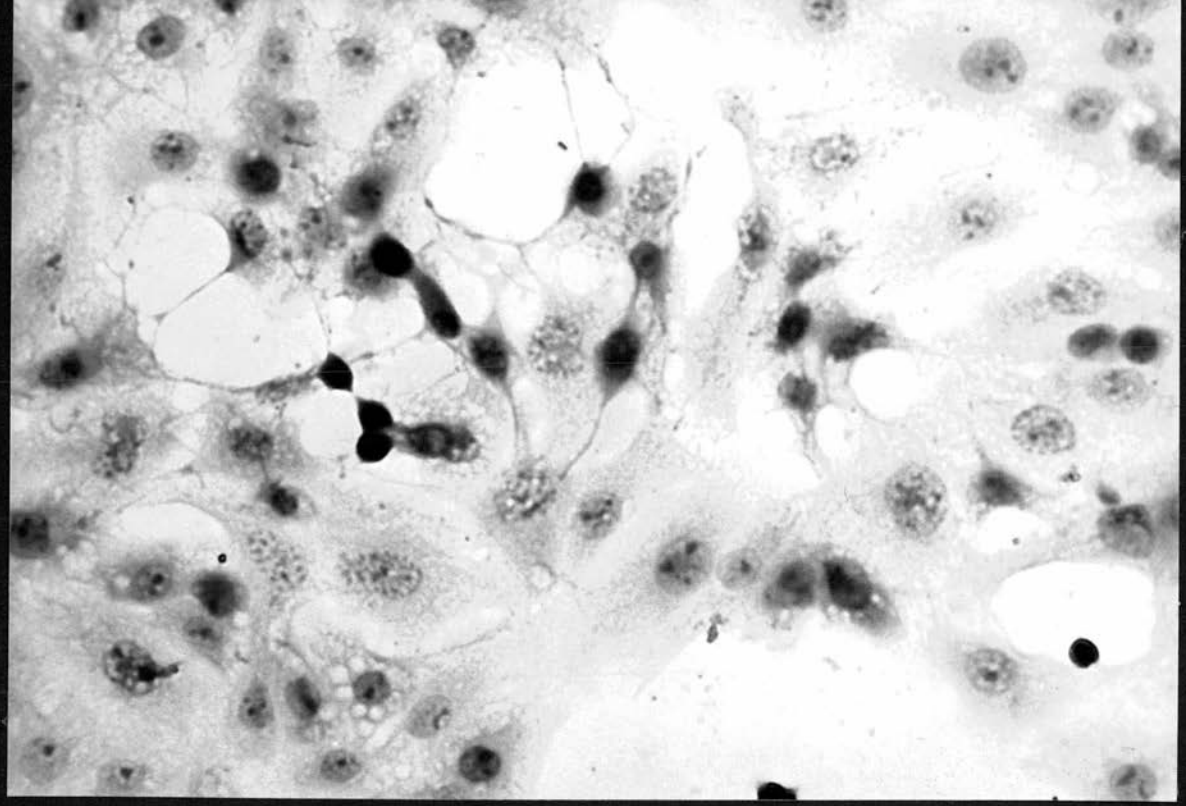
Fig. 21. - Formation of large syncytia containing nuclear inclusions in PK15 cells 12 hours after infection with the McFerran strain of Aujeszky's virus. H & E. x 100.

Fig. 22. - Higher magnification of the above illustrating the nuclear inclusions of Cowdry type A. H & E x 200.





Figs. 23 and 24. - Cytopathic effects of the Hungarian strain of Aujeszky's virus at 36 hours post-infection in PK15 cell cultures; consisting of rounded, shrunken, hyperchromatic cells, small polykaryocytes and nuclear inclusions. x 100.



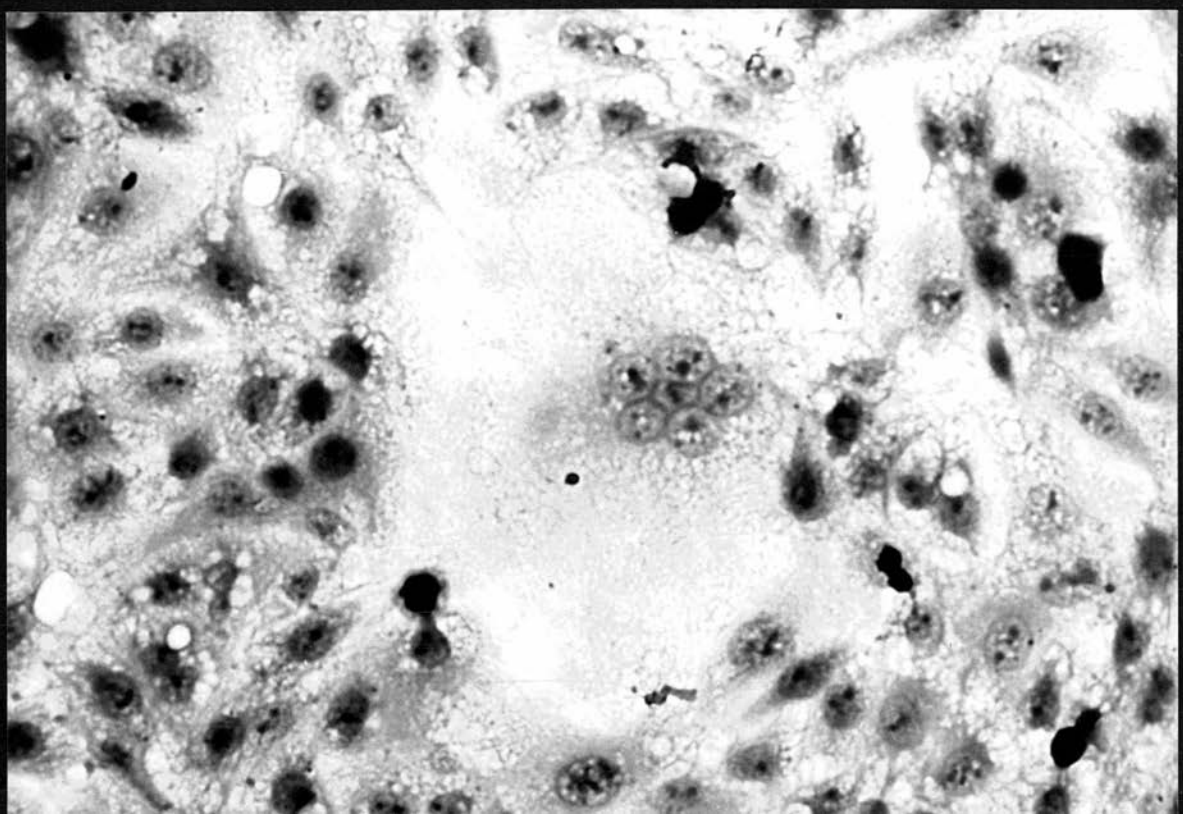


Fig. 25. - The relationship between the age of the cells and the mean day of onset of CPE in PK15 cell cultures infected with the McFerran strain of Aujeszky's virus.

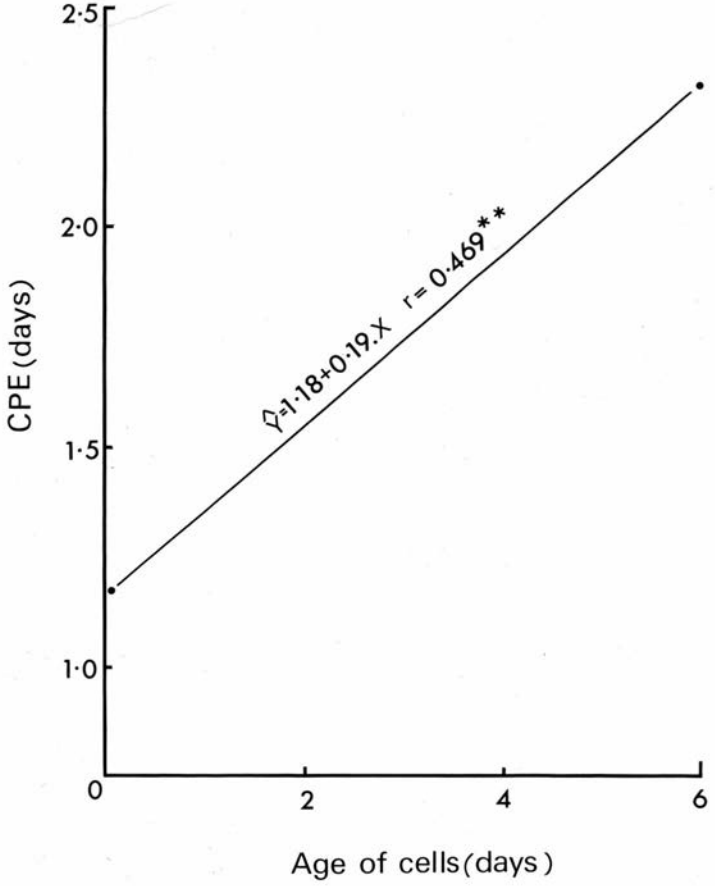

2·5
2·0
1·5
1·0
0
2
4
6
CPE (days)
Age of cells (days)
Ŷ=1·18+0·19.X r= 0·469**

Fig. 26. - The relationship between the dose of the McFerran and the Hungarian strain of Aujeszky's virus on the mean day of onset of CPE in PK15 cell cultures.

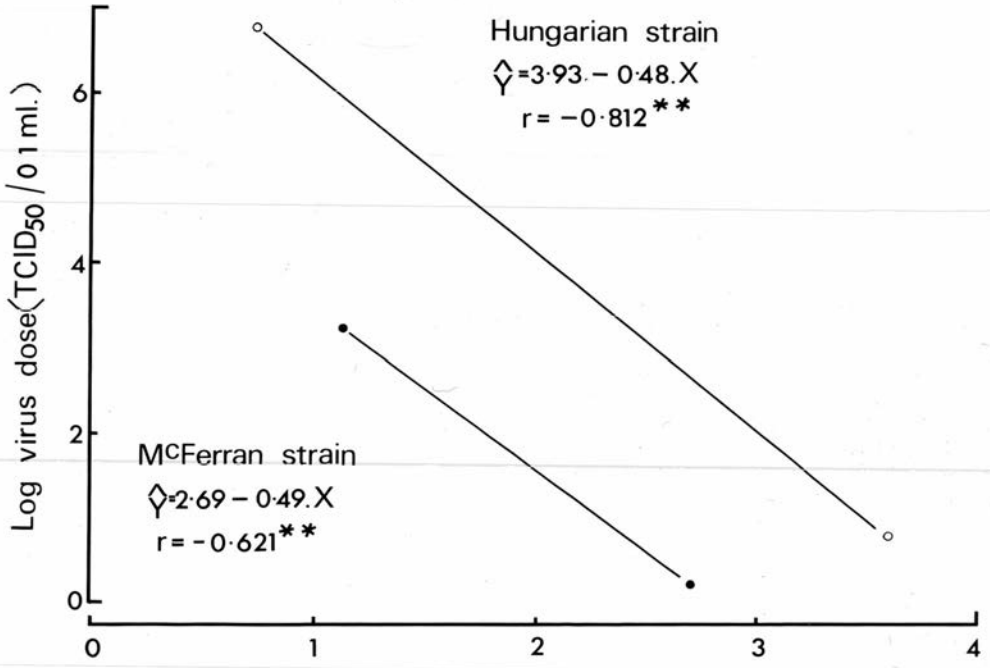
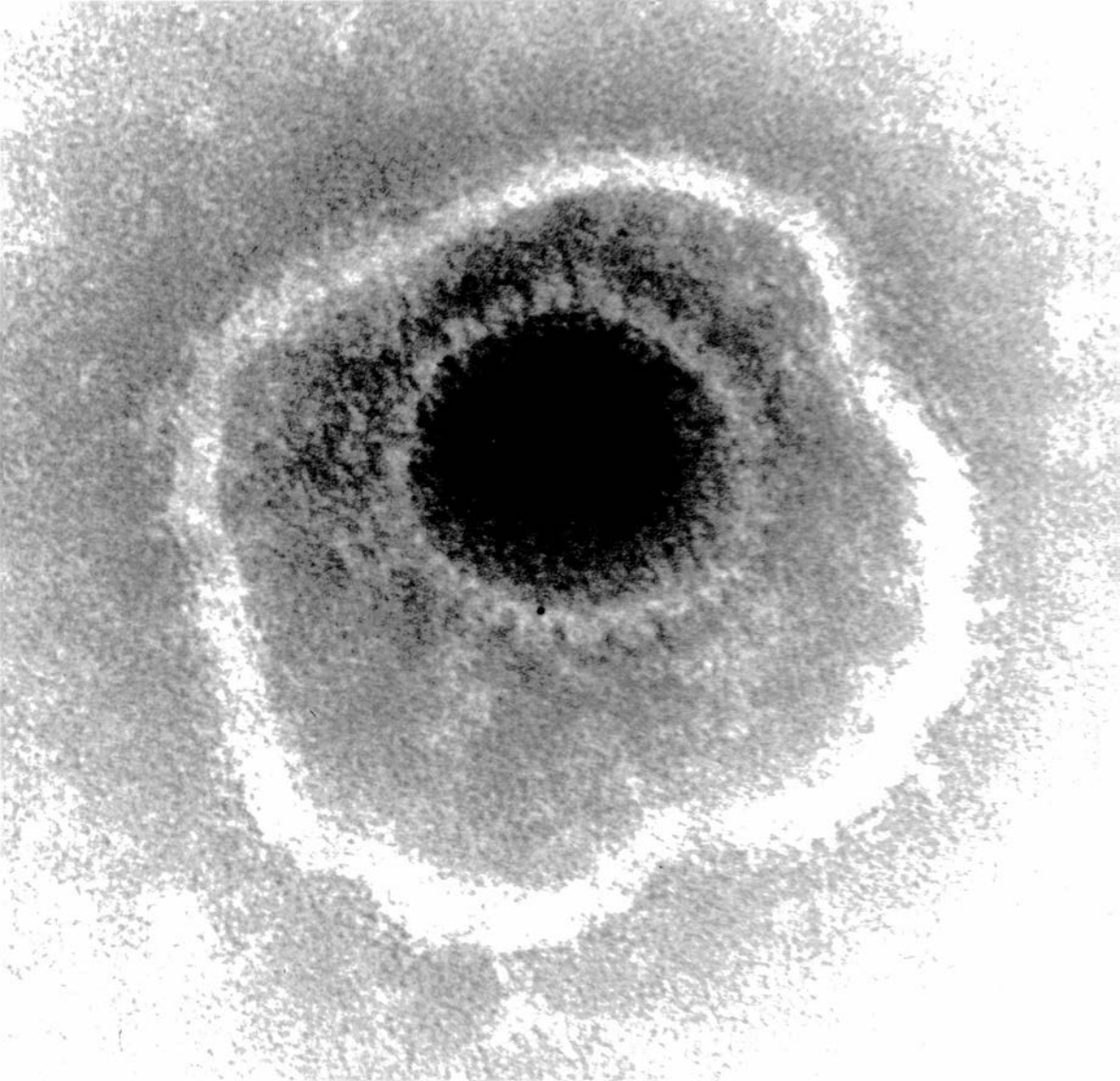

Hungarian strain
$\hat{Y}$=3·93.– 0·48.X
r= –0·812**
McFerran strain
$\hat{Y}$=2·69 – 0·49.X
r= – 0·621**
Log virus dose(TCID50 /0 1 ml.)
Days PI
0
2
4
6
0
1
2
3
4

Fig. 27. - Preparation from PK15 cells infected with the McFerran strain of Aujeszky's virus negatively stained with phosphotungstic acid. The stain has penetrated inside the core giving the capsid an "empty" appearance.

The shell of the capsid consists of a regular radial arrangement of hollow capsomeres. The thick outer membrane seen surrounding the capsid is the "envelope". x 520,000.



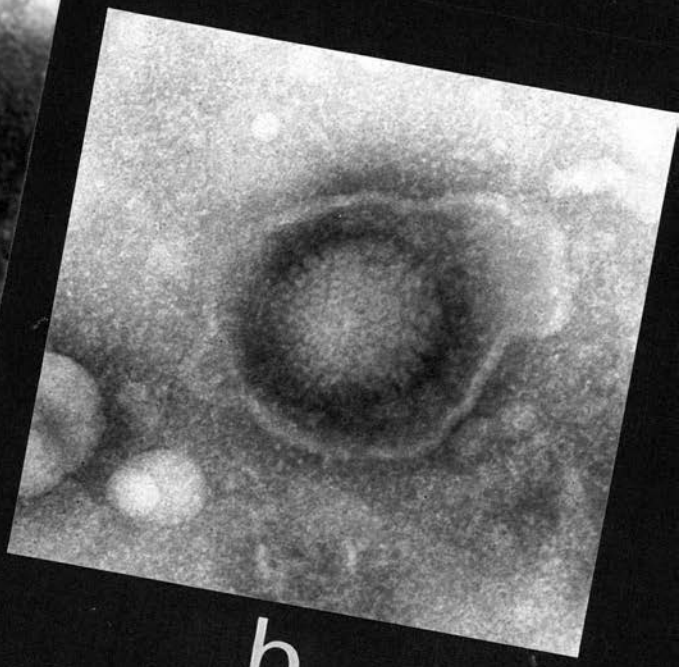
Electron micrographs of the McFerran strain negatively stained with phosphotungstic acid.

a = Fig. 28. - Showing the outer envelope surrounding an 'empty' or "immature" capsid. x 240,000.

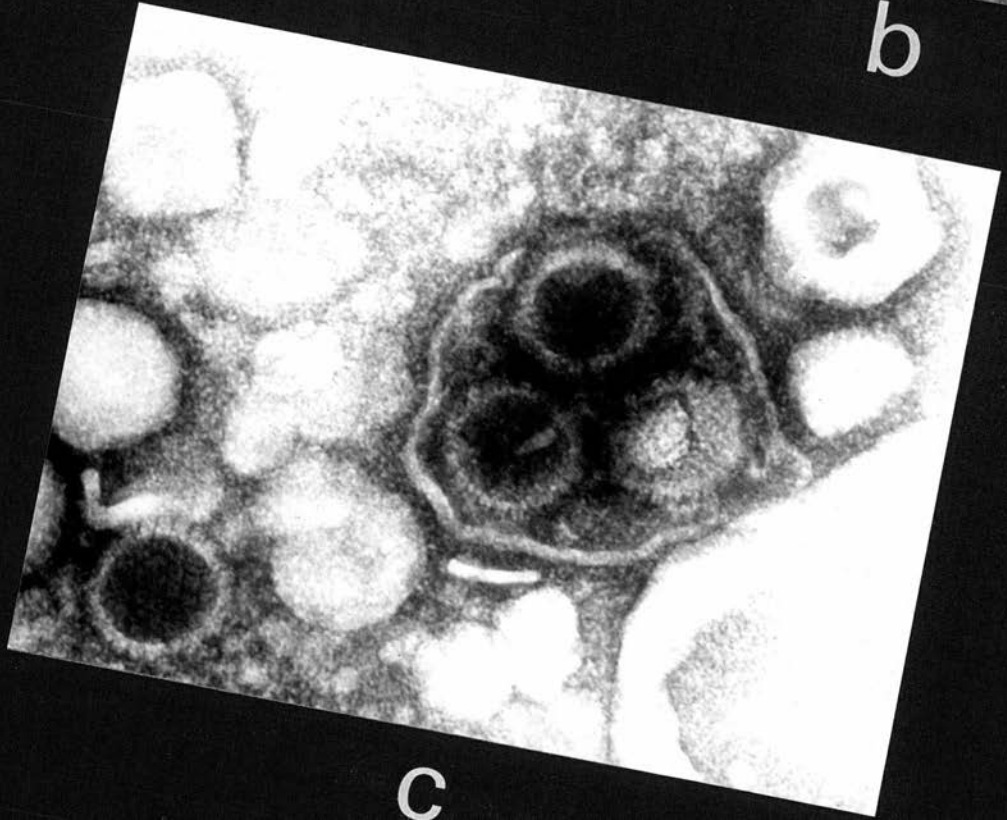
b = Fig. 29. - A "mature" enveloped virion showing the regular arrangement of the surface capsomeres the majority of which are hexagonal in cross section while others are pentagonal. x 200,000.

c = Fig. 30. - Showing three virus capsids within a single envelope. x 160,000.

a

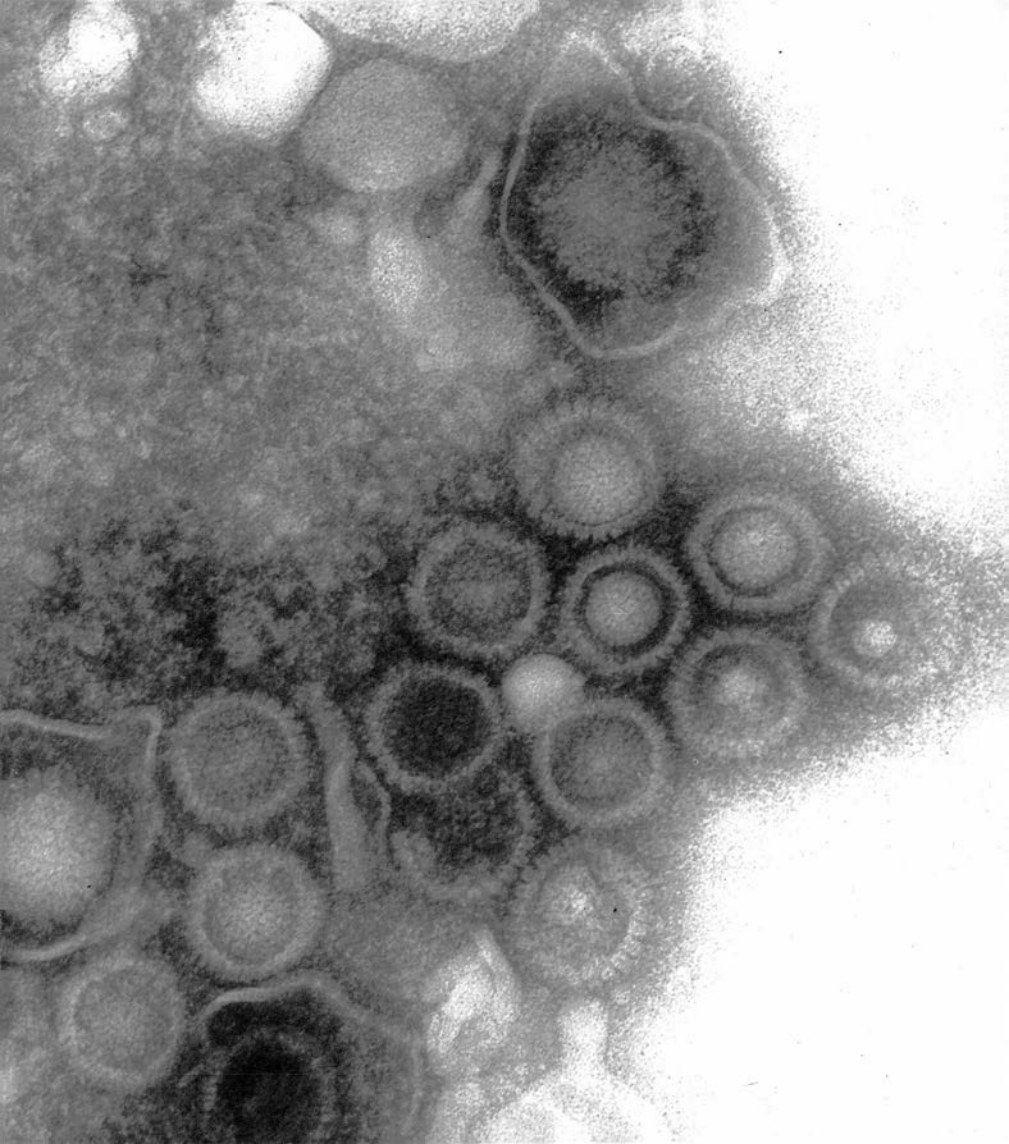


b

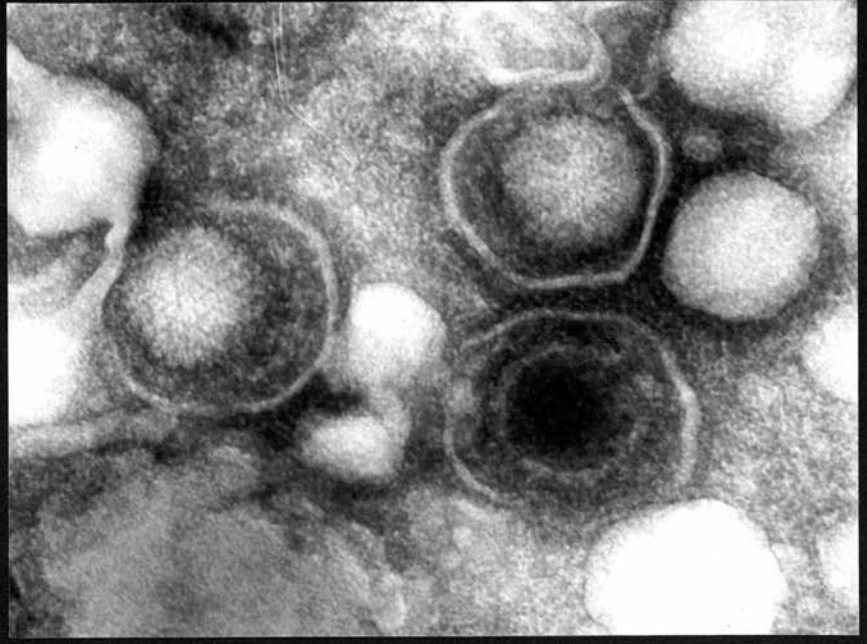


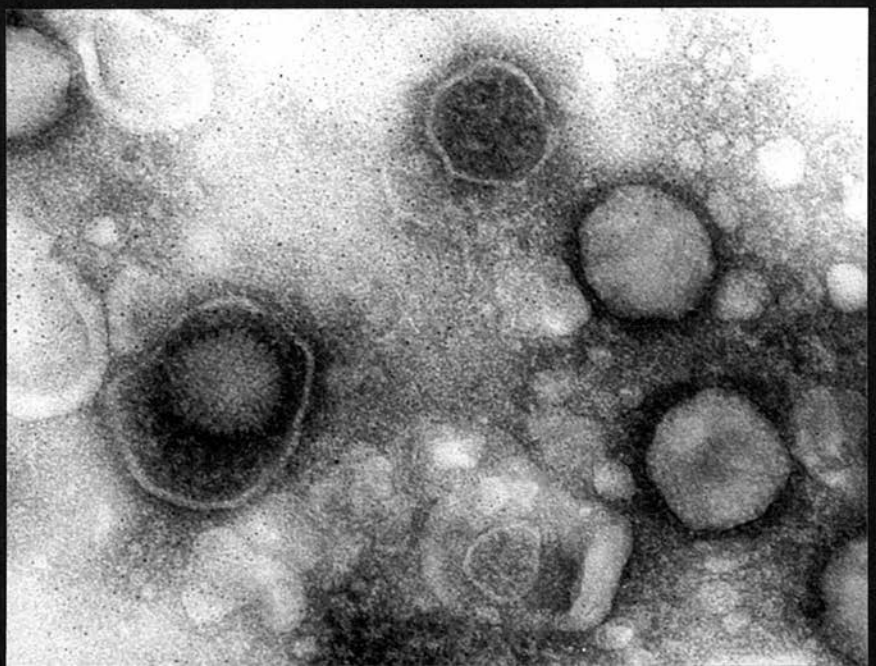
c

Fig. 31. - A group of enveloped and non-enveloped virus particles from a negatively stained preparation of PK15 cells infected with the McFerran strain of Aujeszky's virus. Note the regular arrangement of the capsomeres which appear as hollow hexagonal or pentagonal prisms. x 160,000.



Figs. 32 and 33. - Both illustrations show three capsids in different stages of development each within a clearly defined outer envelope. Two of the capsids reveal icosahedral symmetry and the edge of each triangular facet consists of five capsomeres. x 140,000.





Figs. 34. - Non-enveloped virus particles each showing a distinct "halo" between the outer shell of the capsid and the electron dense core. x 180,000.

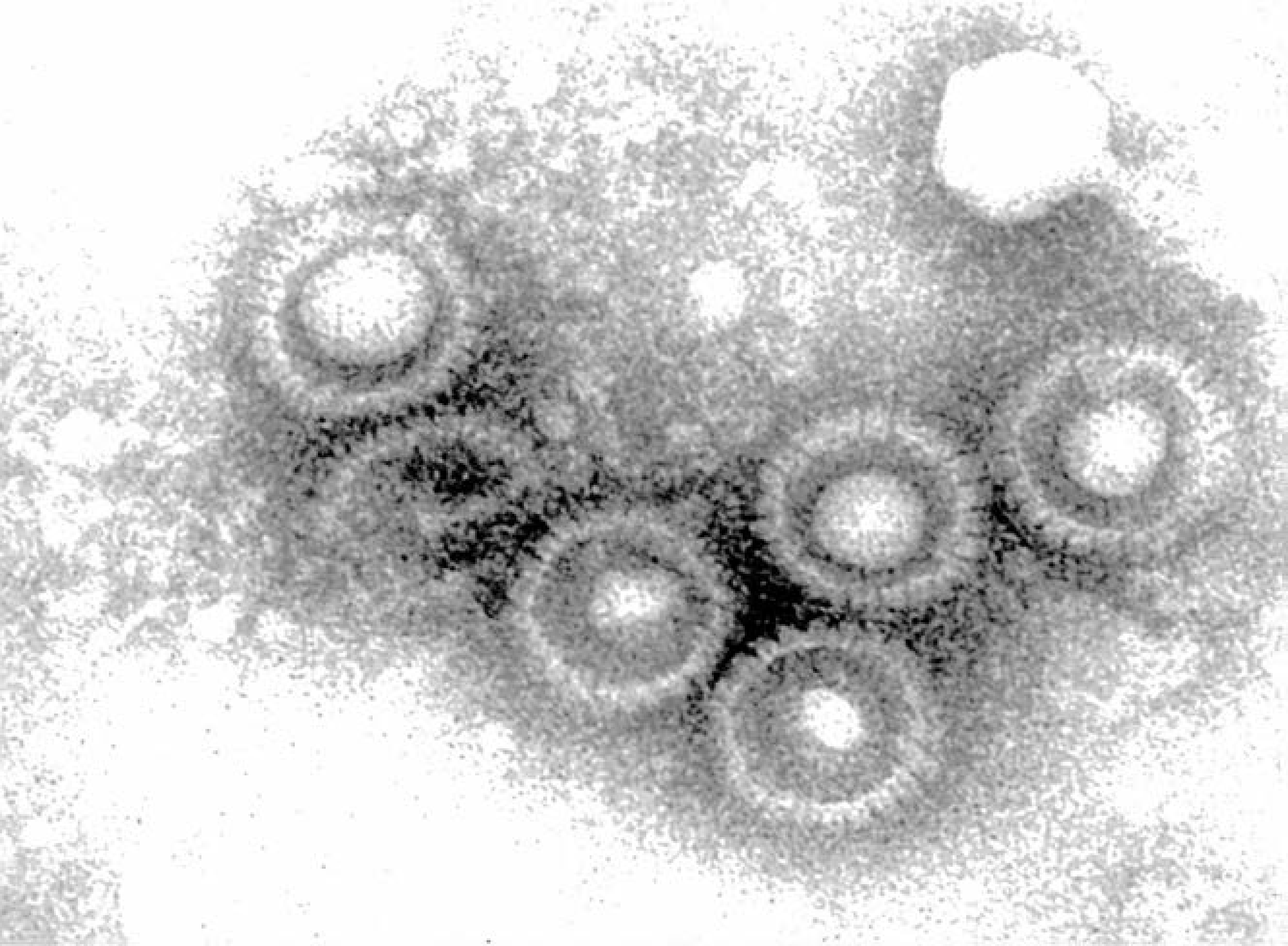
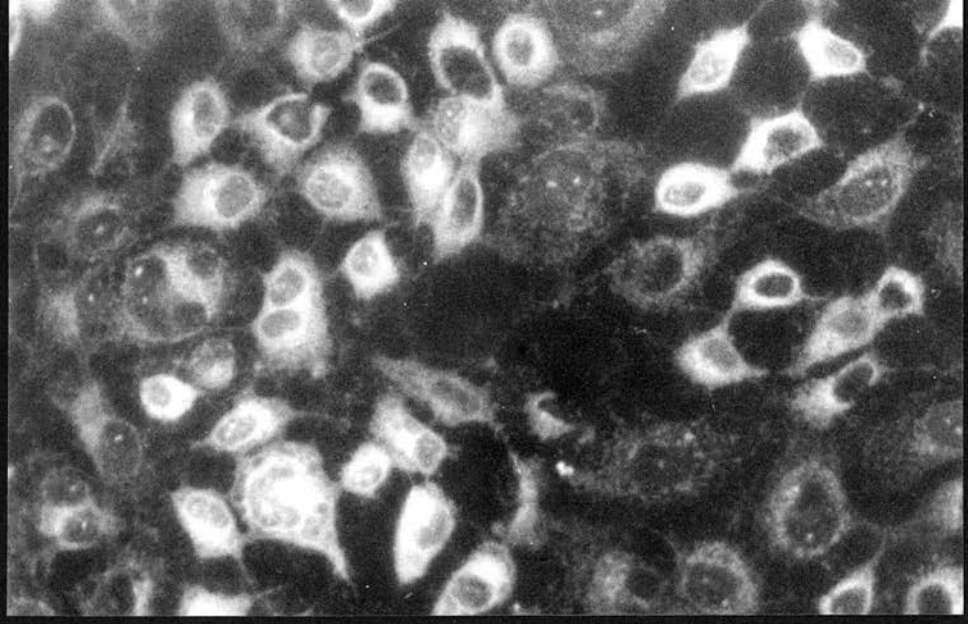


Fig. 35. - Focus of fluorescent cells in PK15 cell culture 4 hours after infection with a high dose of the McFerran strain of Aujeszky's virus showing fluorescence of the nuclear membrane and accumulation of fluorescent material in the perinuclear zone. x 1000.

Fig. 36. - Higher magnification of an area in the same culture showing concentration of particulate fluorescent material in the perinuclear zone on one side of the external nuclear membrane. Some cells show tiny foci of fluorescence in the nucleoplasm. x 2,400.



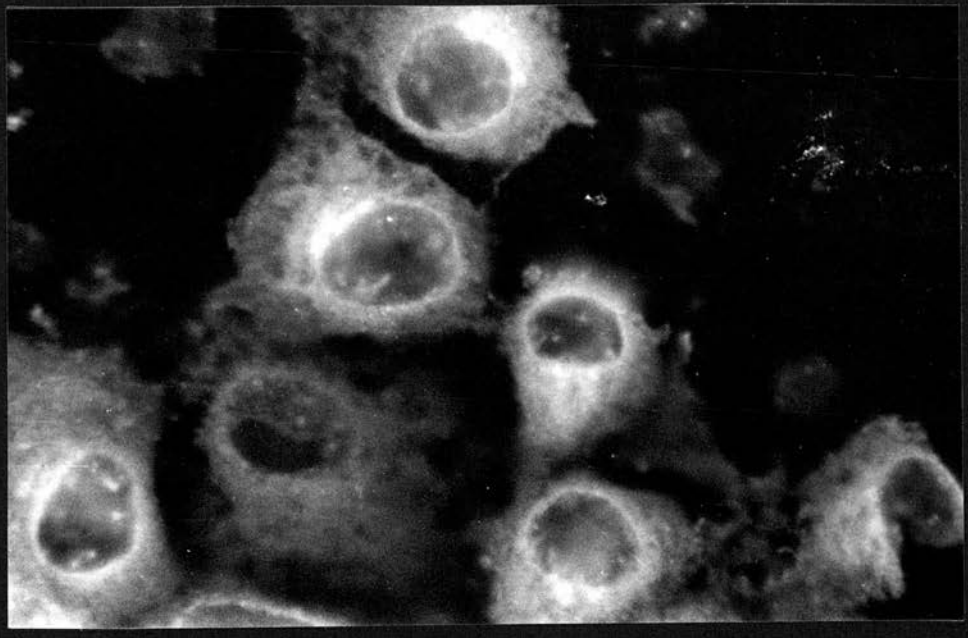
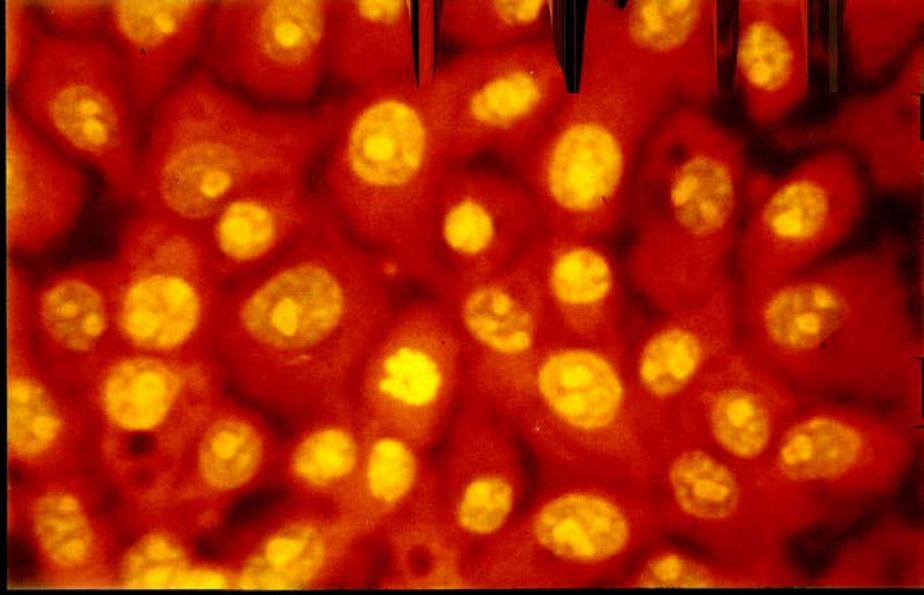


Fig. 37. - Uninfected PK15 cells stained by acridine orange. x 1,500.

Fig. 38. - PK15 cells stained with acridine orange 4 hours after infection with a high dose of the McFerran strain of Aujeszky's virus. Two of the cells show distinct swelling and migration of nucleoli, disintegration of chromatin and the development of greenish-yellow fluorescence in the nuclear matrix. x 2,400.



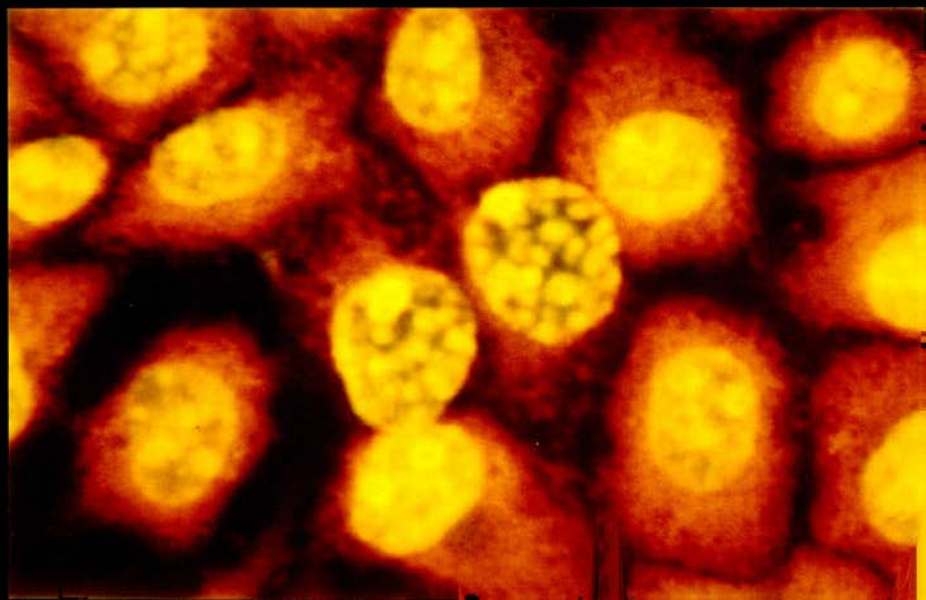
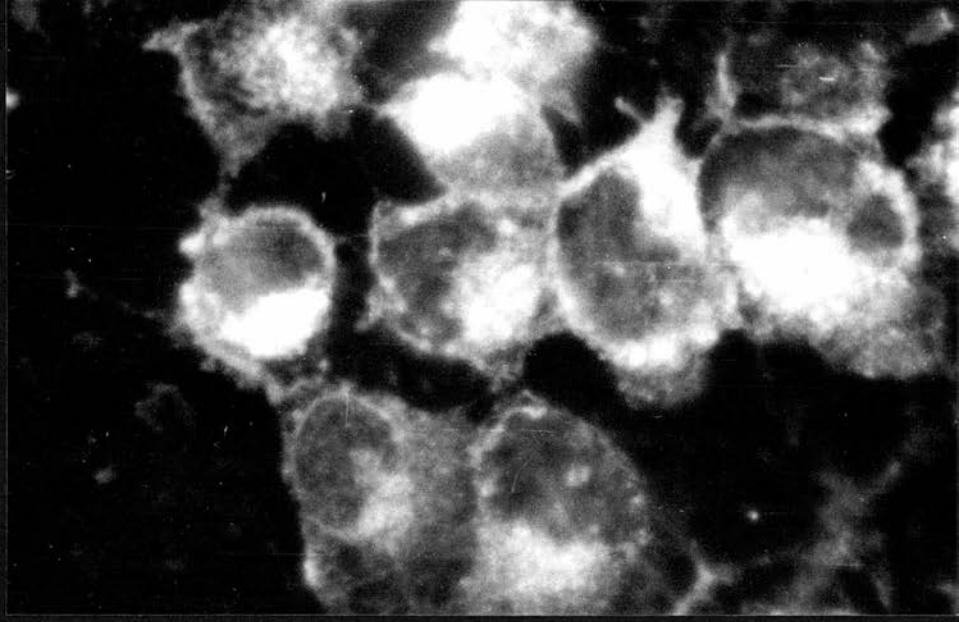
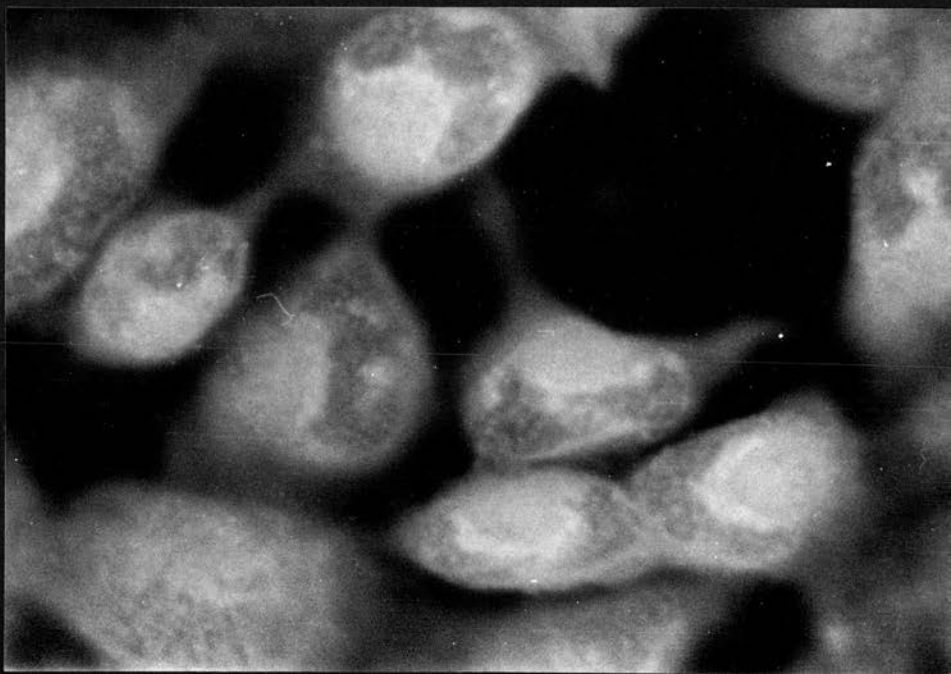


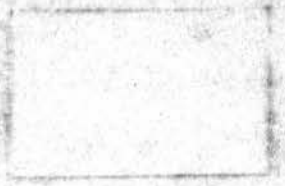
Fig. 39. - Perinuclear and paranuclear deposits of particulate fluorescent material in PK15 cells 8 hours after infection with the McFerran strain of Aujeszky's virus. x 2,400.

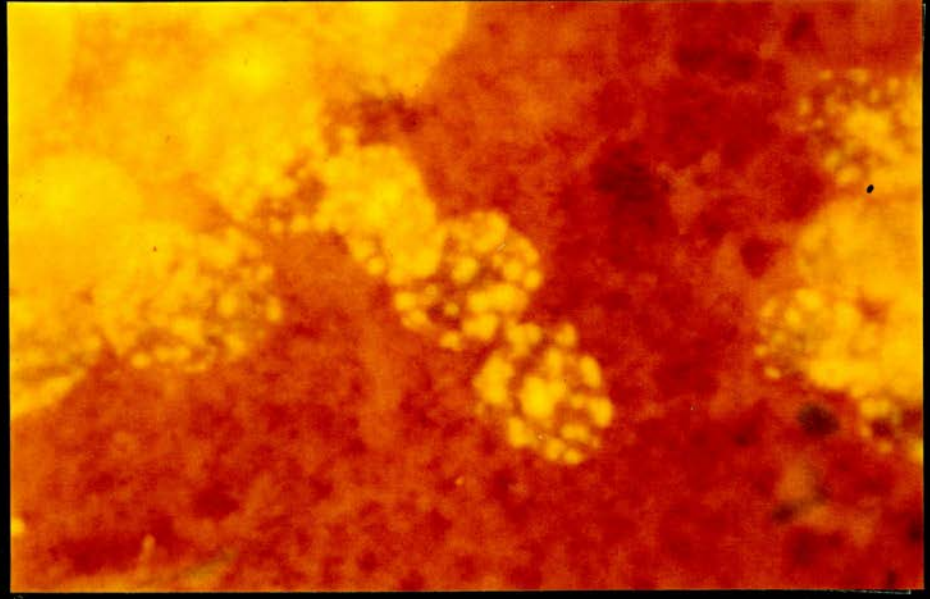
Fig. 40. - Diffuse cytoplasmic fluorescence in PK15 cells infected with the McFerran strain of Aujeszky's virus: 12 hours post-infection. x 2,400.





Figs. 41 and 42. - Syncytia in PK15 cell cultures stained with acridine orange 8 and 12 hours respectively after infection with the McFerran strain of Aujeszky's virus showing breakdown of nuclear structure and abundance of DNA fluorescence. x 2,400.





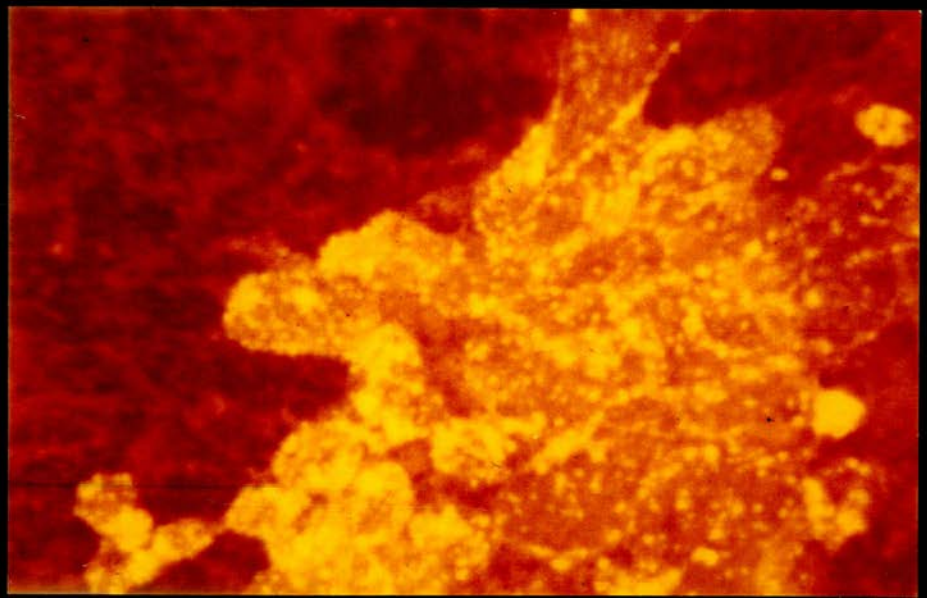
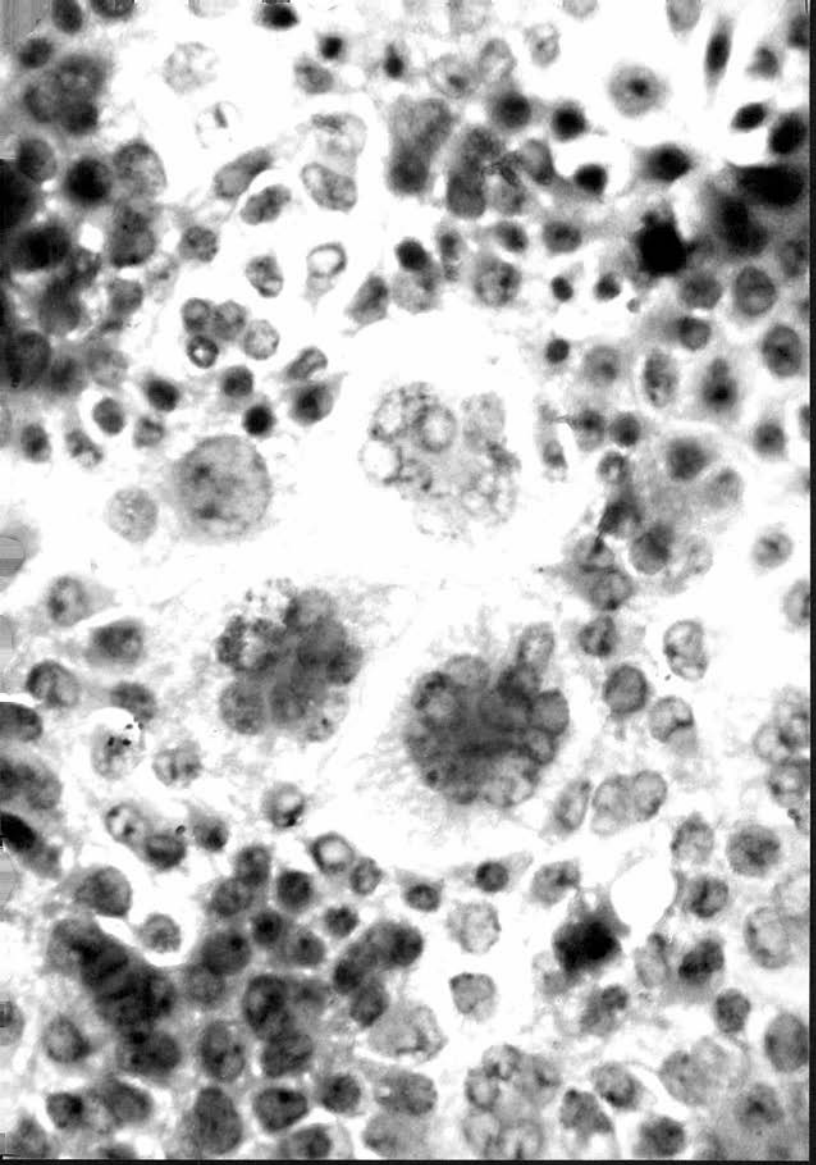


Fig. 43. - Development of small clusters of polykaryocytes in PK15 cells 10 hours after infection with a high dose of the McFerran strain of Aujeszky's virus, H. & E. x 100.

Fig. 44. - Another area in the same culture, showing cells with pyknotic and hyperchromatic nuclei and dispersion of the chromatin. H. & E. x 200.



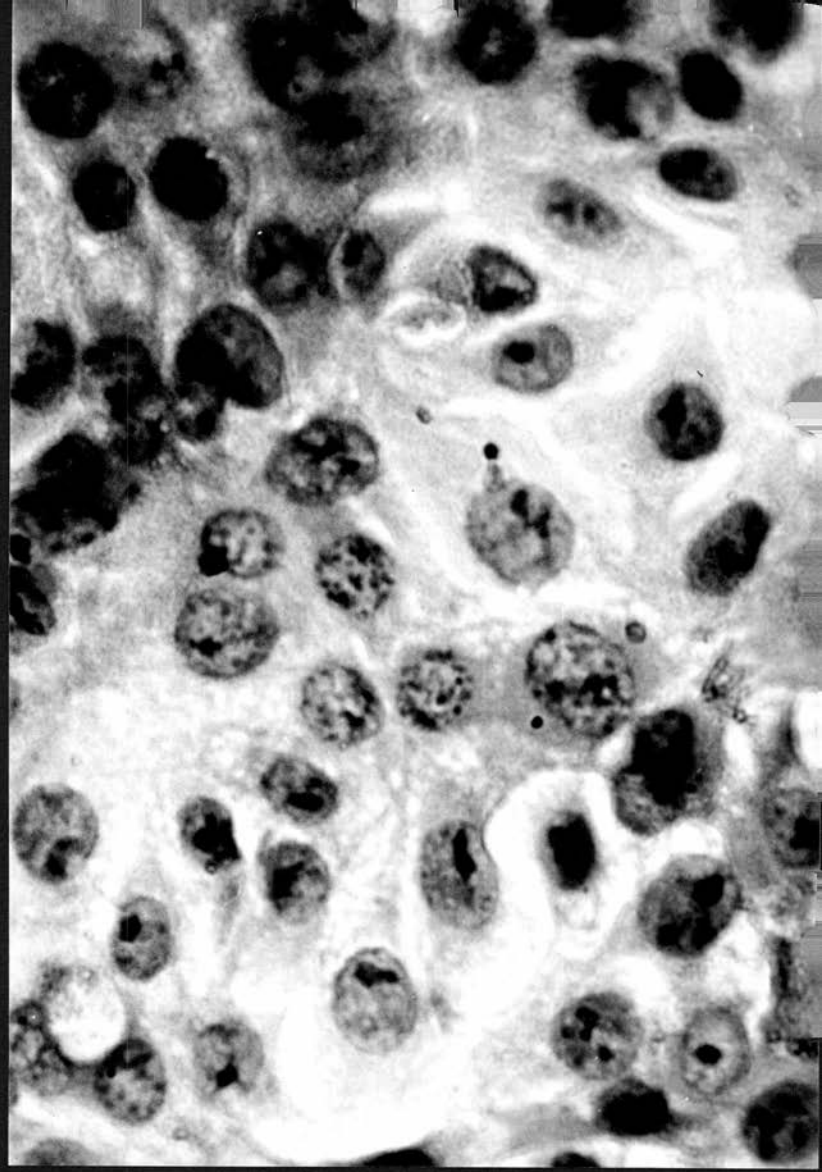


Fig. 45. - Cowdry A type nuclear inclusions in PK cells 12 hours after infection with Aujeszky's virus H. & E. x 200.

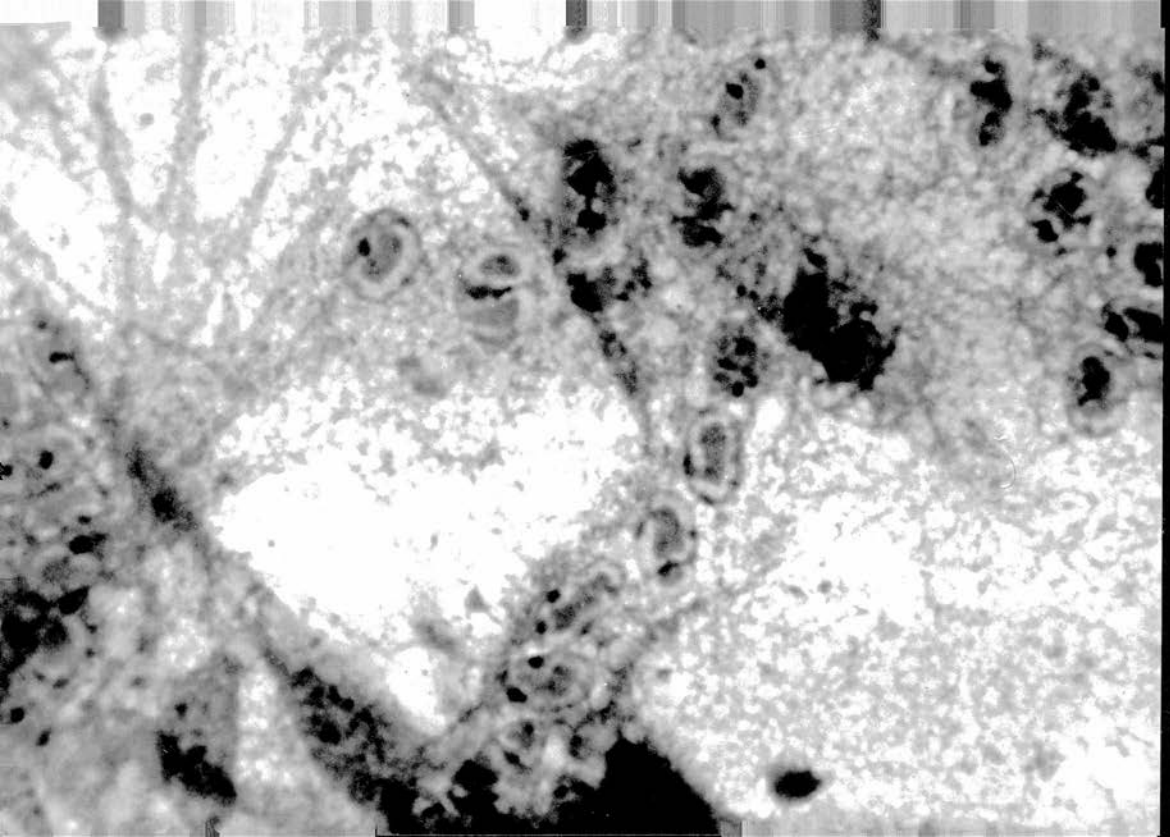


Fig. 46. - Brilliant DNA fluorescence in the nuclei of syncytia in infected RK13 cells 22 hours post-infection. Acridine orange staining. x 1,500.

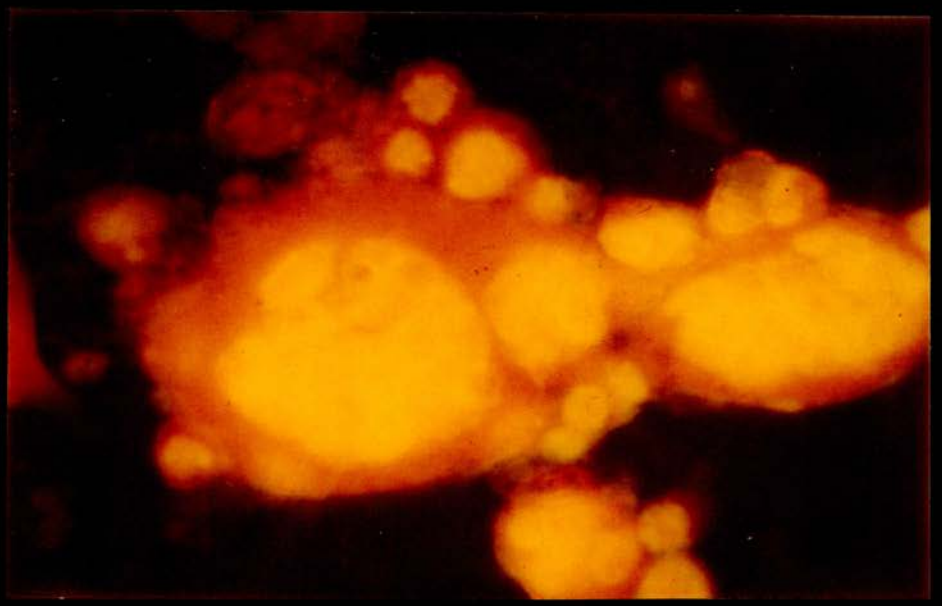
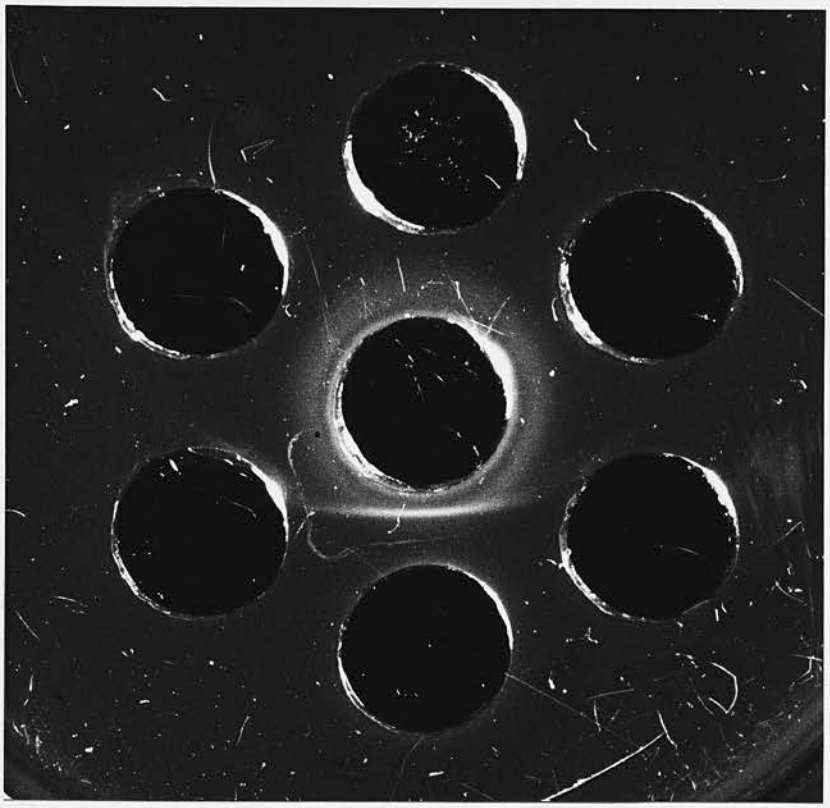


Fig. 47. - A single line of precipitation between concentrated pig anti-Aujeszky's disease hyperimmune serum (central well) and Aujeszky's virus ID antigen (bottom well). The antigen was obtained by treatment of the virus-infected PK15 cell culture fluid with ammonium sulphate. The top and lateral wells contained concentrated culture fluid from uninfected cell cultures.

Fig. 48. - Precipitation lines between reference serum and concentrated culture fluid from PK15 cell culture infected with the virus. The serum was placed in the central well and the undiluted antigen in the top well. Dilutions of the antigen ranging from 1:2 to 1:32 were placed in the right lateral wells, the bottom well and the left lateral wells respectively.



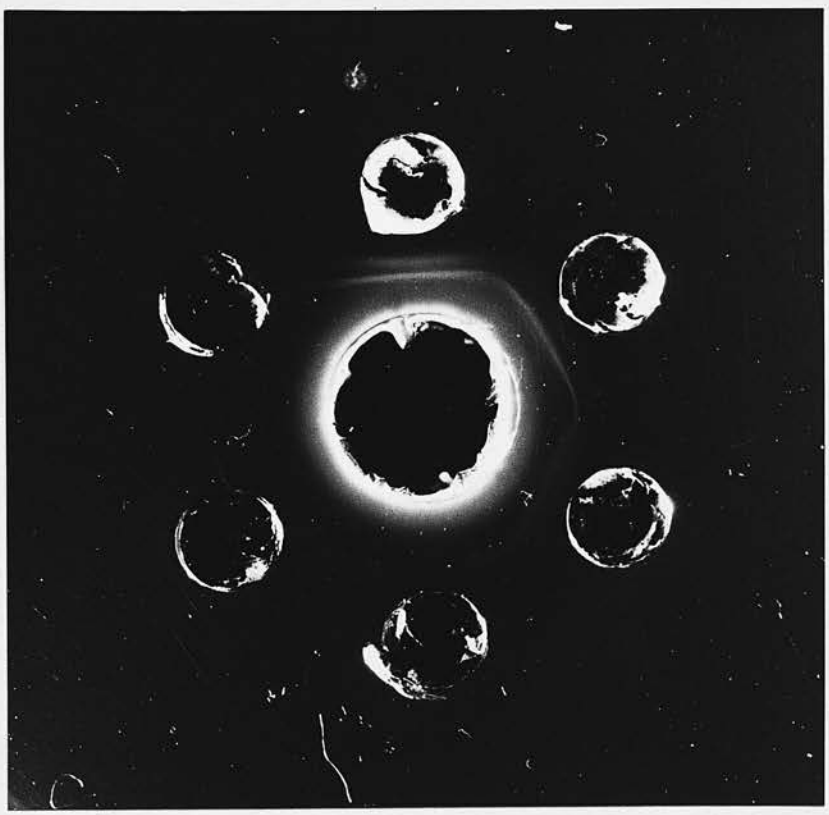
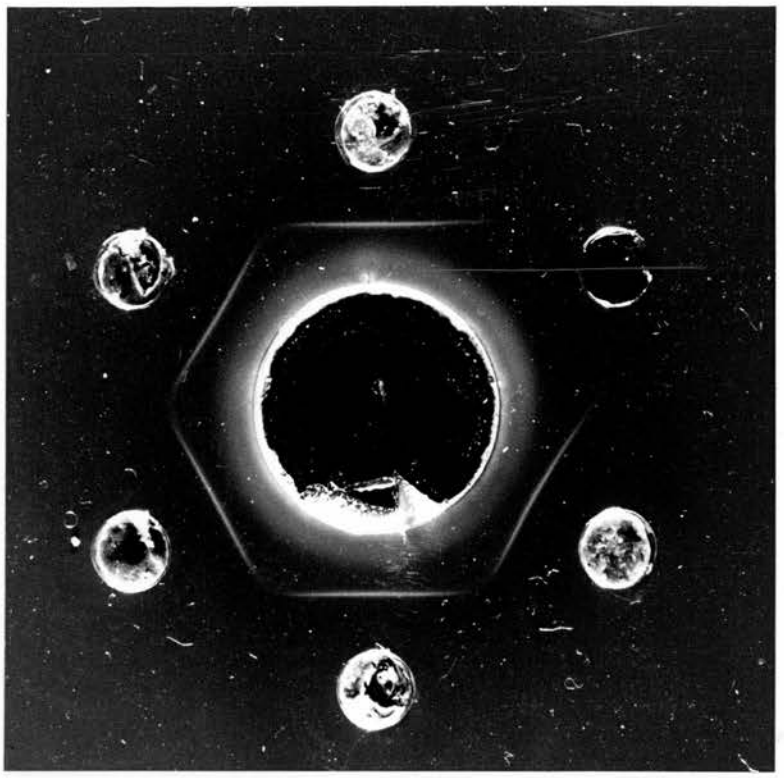


Fig. 49. - Single line of precipitation between reference serum and Aujeszky's virus ID antigen derived from PK15 cells. The central well contained the serum and the peripheral wells, except the upper right lateral well, contained the antigen. Normal saline solution was placed in the upper right lateral well.

Fig. 50. - Emergence of an additional precipitation band in the same plate on further incubation at 37°C. The inner precipitation line appears to have 2 components at the points of union of the contiguous lines.



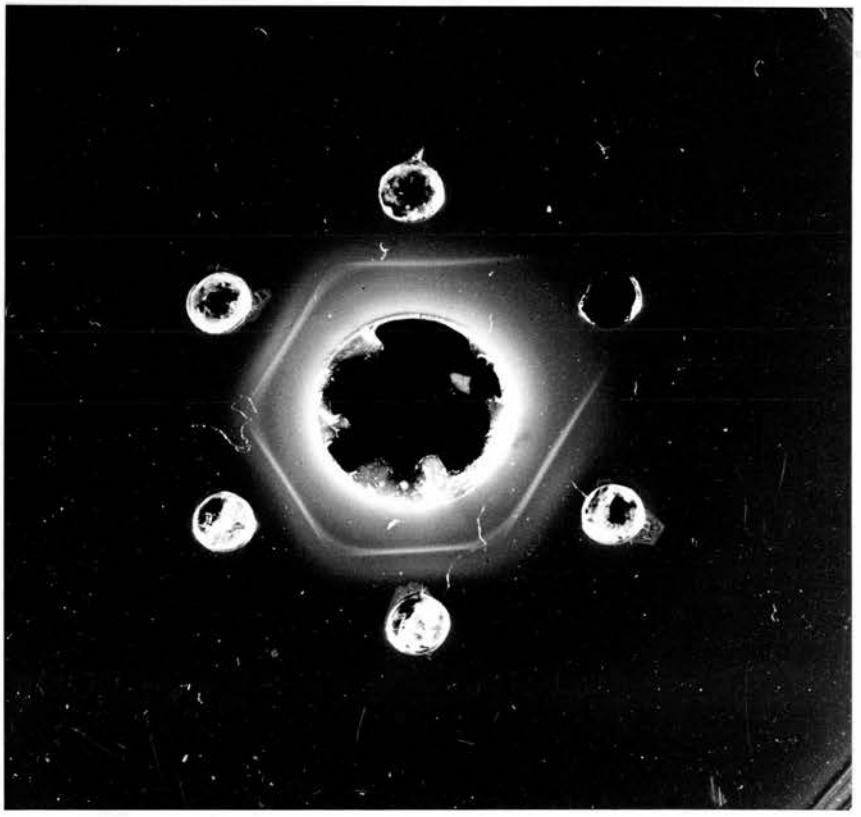
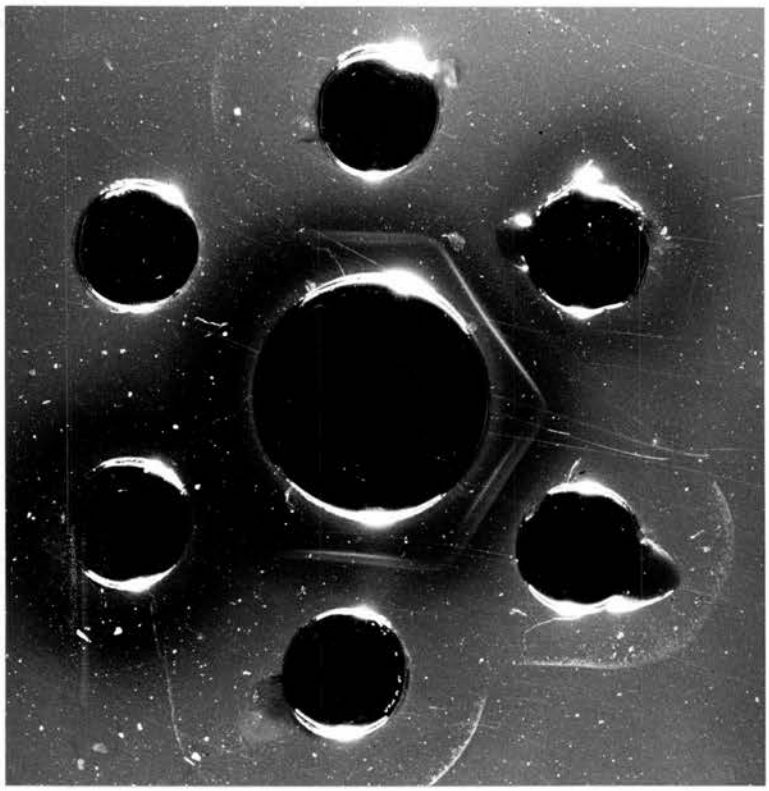


Fig. 51. - Precipitation line between reference serum and Aujeszky's virus ID antigens from infected cultures of different cell types. The central well contained the serum. The upper right lateral well contained antigens derived from infected fowl embryo fibroblasts and culture fluids. The lower right and the lower left lateral wells contained concentrated cell culture fluid from infected PK15 cells. The upper left lateral well contained antigens derived from RK13 cells and the culture fluid. In the top and bottom wells were placed sonicated infected RK13 and PK15 cells respectively.

Fig. 52. - Single and double precipitation lines between the virus ID antigens and the reference serum. Antigens derived from infected RK13 cells and the culture fluid (upper left lateral well) and infected fowl embryo fibroblasts and culture fluid (top well) produced 2 lines of precipitation with the serum; the contiguous lines merged indicating a reaction of identity. Likewise, the antigens from infected PK15 cells and culture fluids (lower left lateral well) and sonicated RK13 cells and culture fluid (bottom well) diffused towards the antiserum producing a reaction of identity even after exposure to 60°C for 30 minutes. The upper right lateral well contained uninfected cell culture fluid and the lower right lateral well contained infected MDBK cells and culture fluids.



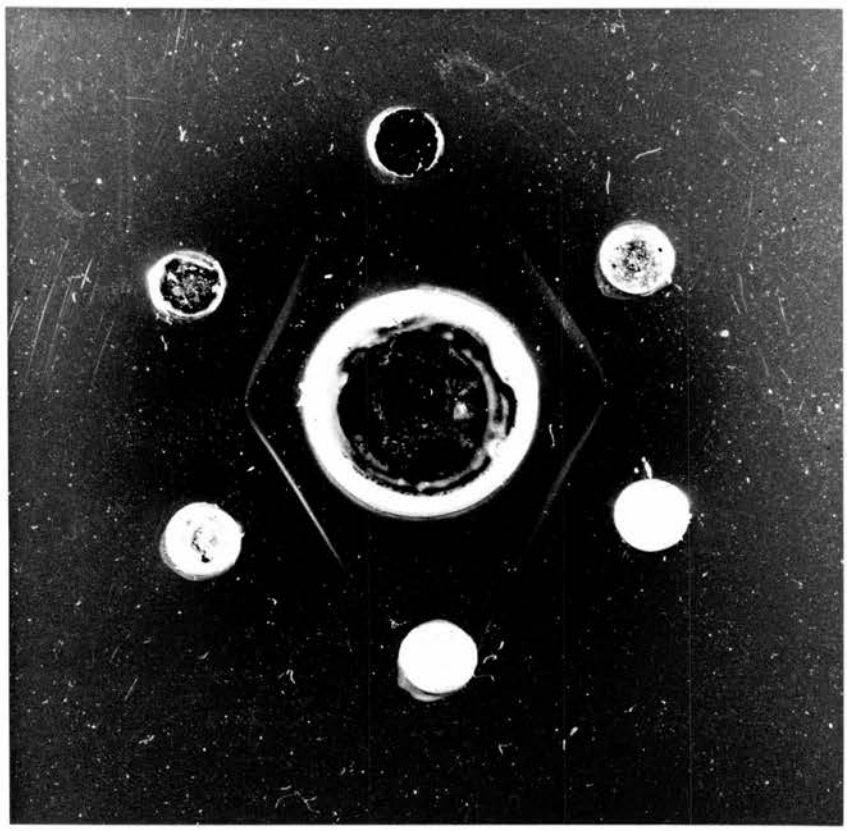


Fig. 53a. - Precipitation reactions between Aujeszky's virus ID antigens (central well) and reference serum that was concentrated by dialysis against carbowax (top and bottom wells). The lateral wells contained concentrated supernatant fluid obtained after precipitation of the reference serum with ammonium sulphate.

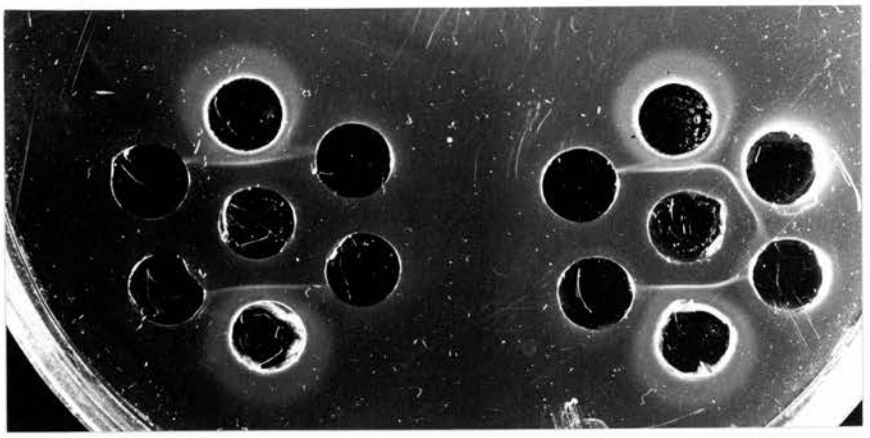
Fig. 53b. - Precipitation lines between the virus ID antigens (central well) and reference serum that was concentrated by precipitation with ammonium sulphate. The undiluted precipitated fraction was placed in the upper right lateral well. The lower right lateral well, and the lower and upper left lateral wells contained 1:2, 1:4 and 1:8 dilutions of the fraction respectively. The top and bottom wells contained the reference serum that was concentrated by dialysis against carbowax.

Fig. 54a. - The top and bottom wells contained the reference serum. The upper left and lower right lateral wells contained two different batches of Aujeszky's virus ID antigens derived from RK13 cells. The central well contained concentrated uninfected cell culture medium. The upper right and the lower left lateral wells contained chicken anti-Aujeszky's disease sera. Two to 3 lines of precipitation occurred between the reference serum and the antigens, but there was no reaction between the chicken sera and the antigens.

Fig. 54b. - Reaction specificity of the virus ID antigens derived from RK13 infected cell cultures (right and left lateral wells) and the reference serum (central well). The top and bottom wells contained concentrated, uninfected cell culture fluid.

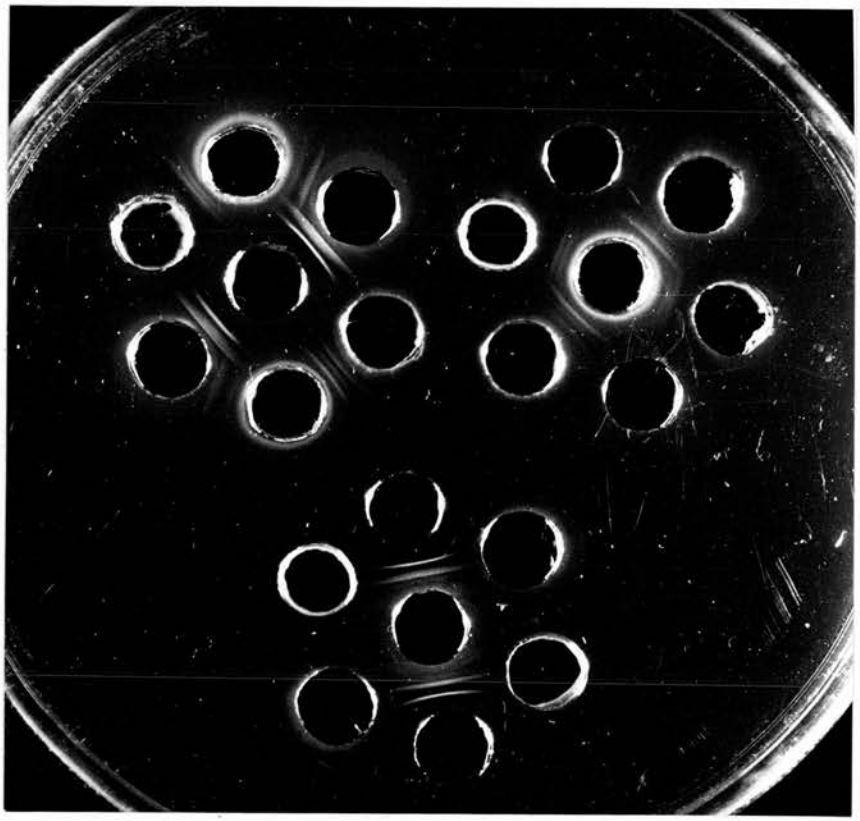
Fig. 54c. - Absence of specific immunodiffusion antibodies in chicken anti-Aujeszky's disease serum. The serum was placed in the central well. The top and bottom wells contained concentrated, uninfected cell culture medium. The lateral wells contained four different batches of Aujeszky's virus ID antigens.

a



b

a



b

c

PATHOGENICITY FOR FOWL EMBRYOS

The pathogenicity of the McFerran strain of Aujeszky's virus for the fowl embryo was investigated by injecting seven days old embryos into the yolk sac and 10 days old embryos into the allantoic cavity or on to the chorioallantoic membrane. Two series of parallel titrations were carried out using cell culture virus pools that had infectivity titres of $10^{7.7}$ and $10^{8.7}$ $TCID_{50}$ per ml. respectively.

In general, fowl embryos infected with Aujeszky's virus showed congestion of blood vessels, haemorrhage in the brain, diffuse anasarca and hydrocephalus. In addition, embryos injected via the yolk sac showed pronounced congestion and oedema of the yolk sac membrane, whereas those inoculated on the CAM manifested changes ranging from a general thickening and oedema of the membrane to the presence of discrete or coalescent greyish or whitish necrotic foci, the so-called 'pocks'. For the determination of LD_{50} values embryonic death was used as the main guide line.

Analysis of the responses of the embryos to infection by the yolk sac, CAM and allantoic routes revealed significant differences in the LD_{50} values of the virus, the yolk sac route being the most effective (Tables 25 and 26). The mean death periods following allantoic, chorioallantoic and yolk sac injections were

5.90 ± 0.18, 4.38 ± 0.26 and 3.86 ± 0.17 respectively; and the differences in the duration of the allantoic and CAM infections and of the allantoic and yolk sac infections were significant (Table 26). Whichever route of infection was used, the mean period of death of the embryos was inversely related to the dose of virus given such that higher doses induced mortalities earlier than lower doses, and the lines of best fit were linear and significant (Table 27; Fig. 55).

TABLE 25

LD_{50} OF THE McFERRAN STRAIN OF AUJESZKY'S VIRUS FOR FOWL EMBRYOS INFECTED BY DIFFERENT ROUTES

Route of inoculation	LD_{50} + S.E.	
	Experiment 1	Experiment 2
Yolk Sac	$\frac{}{10}$ 6.20 ± 0.36	$\frac{}{10}$ 7.20 ± 0.45
Allantoic cavity	$\frac{}{10}$ 2.45 ± 0.58	$\frac{}{10}$ 5.38 ± 0.40
Chorioallantoic membrane	$\frac{}{10}$ 5.90 ± 0.30	$\frac{}{10}$ 5.70 ± 0.43

TABLE 26

COMPARISON OF THE MEAN RESPONSES OF FOWL EMBRYOS INFECTED WITH THE McFERRAN STRAIN OF AUJESZKY'S VIRUS

Parameter	Route of infection	Difference	S.E. of differences	d.f.	t	P
LD_{50}	Yolk sac and allantoic cavity	$10^{-1.82}$	0.601	16	3.028**	<0.01
	Yolk sac and CAM	$10^{-1.50}$	0.622	16	2.412**	<0.01
	CAM and allantoic cavity	$10^{-0.32}$	0.587	16	0.545	70.05
Mean death period	Allantoic cavity and CAM	1.52	0.382	67	3.963**	<0.01
	Allantoic cavity and Yolk sac	2.04	0.275	78	7.418**	<0.01
	CAM and Yolk sac	0.52	0.300	93	1.733	>0.05

TABLE 27

REGRESSIONS OF THE MEAN DAYS OF DEATH IN FOWL EMBRYOS ON THE DOSE OF AUJESZKY'S VIRUS INOCULATED BY DIFFERENT ROUTES

Route of Infection	Regression	d.f.	Correlation coefficient
Allantoic Cavity	$\hat{Y} = 7.03 - 0.23.X$	25	0.377*
CAM	$\hat{Y} = 7.27 - 0.65.X$	40	0.632**
Yolk Sac	$\hat{Y} = 5.85 - 0.50.X$	51	0.599**

Fig. 55. - The relationship between the dose of virus and the mean day of death in fowl embryos infected on the chorioallantoic membrane (right) or into the yolk sac (left) with the McFerran strain of Aujeszky's virus.

Virus dose (log_{10} TCID$_{50}$/0·2ml.)
6
4
2
0
0
2
4
6
Day of death

PATHOGENICITY FOR CHICKENS

CLINICAL SYNDROME

Experimental Aujeszky's disease induced in day-old chicks by different peripheral routes of infection had no distinctive clinical features except those that are commonly associated with nervous injury. Chicks that succumbed to the infection manifested general malaise, anorexia, lassitude, dyspnoea, muscular tremors, incoordination of movement, crouched appearance, progressive generalised paralysis, coma and death (Figs. 56 and 57); but it was often difficult to pinpoint the time of onset of the symptoms. In the terminal stages of the infection, most birds showed stringy salivation from the beak. Paresis of the inoculated leg was a common feature in intramuscularly injected chicks up to 3 days of age but hyperaesthesia of the skin which is pathognomic of the natural disease in ruminants and carnivores and of the experimental infection of rodents was conspicuously absent in infected chicks. Older birds that succumbed to the disease showed similar symptoms.

THE McFERRAN STRAIN

Effect of Route of Inoculation: The susceptibility of young chicks to Aujeszky's virus was investigated by infecting groups of chicks of less than 24 hours of age by various routes with 0.05 to 0.1 ml. amounts of the

McFerran strain of cell cultured virus having an infectivity titre of $10^{7.0}$ $TCID_{50}$ per ml. The results revealed that Aujeszky's virus is pathogenic for chicks less than 24 hours of age and that the mortality pattern is influenced by the route of infection, the intracerebral route being the most effective (Table 28). The mean duration of clinical disease was significantly affected by the route of injection of the virus ($F = 9.59^{**}$, d.f. 5, 45) and chicks injected intranasally, intraocularly or intradermally lived longer than those infected intramuscularly, subcutaneously or intracerebrally. Whereas there were no differences in the mean time of death in the first group of chicks ($F = 2.12$, d.f. 5, 45), the difference in the mean periods of fatal infection in the second group was significant ($F = 4.95^{*}$, d.f. 2, 28). The mean duration of infection was shortest in the intracerebrally infected group.

Death of the inoculated chicks within 10 days was considered as evidence of virus infection and a positive correlation between mortality and virus recovery from the brain was observed even in delayed deaths. Day-old chicks inoculated intracerebrally or intramuscularly with either uninfected tissue culture fluid or heat inactivated virus showed no signs of illness and all succumbed to the infection when challenged intracerebrally

with live virus.

Effect of Age: Because of the high percentage of mortalities obtained in day-old chicks injected by the intracerebral, intramuscular and subcutaneous routes, studies were carried out to determine the extent to which the susceptibility of chickens to Aujeszky's virus might be influenced by factors such as age and dose.

Intracerebral infection: Seven groups of chickens of ages ranging from 1 to 180 days were infected intracerebrally with 0.05 ml. amounts of a virus pool that had an infectivity titre of $10^{7.5}$ $TCID_{50}$ doses per ml. The results of this sighting experiment revealed that age had a marked influence on the percentage and distribution of virus-induced mortalities in chickens (Table 29). The relationship between age and the percentage of mortalities was inverse and significant ($F = 6.92^{*}$ d.f. 2, 4) and the line of best fit was curvilinear rather than linear (Fig. 58). Similarly, there was a direct relationship between the mean time of death and the age at which the chickens were injected, and the relationship was curvilinear and significant ($F = 14.00^{*}$ d.f. 2, 4; Fig. 59).

In general, the influence of age on susceptibility to experimental Aujeszky's disease in the intracerebral

route was more decisive in chickens of 1 to 7 days of age than in birds of older age groups. This was evident in the fairly uniform distribution of deaths in the former group, which was in contrast to the incidence of dealyed deaths and higher percentage of survivals in the older groups of chickens.

The influence of the age of the chickens on susceptibility to experimental Aujeszky's infection was further investigated by the intracerebral inoculation of different age groups of birds with 0.05 ml. of ten-fold dilutions of the same virus pool. The results (Table 30) of this experiment confirmed the previous observation that mortality induced by Aujeszky's virus declined progressively with age. The regression of LD_{50} on age appeared to be curvilinear and the line of best fit was $\hat{Y} = 0.970.X - 0.075.X^2 - 4.910$ where X is age in weeks. The regression (2d.f.) was compared with deviations from regression (3d.f.) in the analysis of variance. The variance ratio (F) was 8.16 which failed to reach the 0.05 (5 per cent) level of significance. However, the differences between the LD_{50} values of Aujeszky's virus for day-old and one week-old chicks and for one week-old and 6 to 7 weeks' old birds were highly significant (Table 31).

Intramuscular infection: Chickens of different ages ranging from 4 hours to 42 days were injected in the thigh muscles with Aujeszky's virus ($10^{7.0}$ $TCID_{50}$ per ml); the very young chicks received 0.1 ml. and those above 7 days of age 0.2 to 0.5 ml. of the inoculum. Mortalities occurred only in chicks of 4 to 37 hours of age (Table 32) and the older birds showed no clinical syndrome. Whereas there was no evident link between the age of the young chicks and the mean duration of fatal infection ($F = 0.25$, d.f. 1, 49), the percentage of deaths was inversely and significantly related to age ($F = 76.07^{**}$, d.f. 1, 3) and the line of best fit was linear (Fig. 60). The apparent age-related resistance of chickens to experimental Aujeszky's infection was further evident in the results of another experiment in which groups of chicks of less than 12 hours to 72 hours of age were inoculated intramuscularly with serial 10-fold dilutions of the virus (Table 33). Whereas chickens of above 57 hours of age or older showed no adverse effects, those of younger age groups showed a range of mortalities that was closely related to age ($F = 21.44^{**}$, d.f. 1, 52), the relationship being negative, linear and highly significant ($\hat{Y} = 136.37 - 7.40.X$; $r = -0.541^{**}$ where X is age, $\hat{Y}$ is the line of best fit and r the correlation coefficient). A comparison of the LD_{50} values of the

virus for 7 to 10 hours old chicks and 13 hours old chicks showed significant differences (Table 34).

Subcutaneous infection: To determine the mortality pattern following subcutaneous inoculation, chickens of different ages were injected in 0.2 ml. amounts with cell culture virus that had an infectivity titre of $10^{7.0}$ $TCID_{50}$ per ml. The results confirmed the observation that the susceptibility of young chicks to Aujeszky's infection declines rapidly with age (Table 35) and it was also interesting to note that some degree of resistance had developed even at 34 hours after hatching. In chicks less than 34 hours old mortalities were related significantly to age ($F = 11.97^{*}$, d.f. 1, 7) and the relationship was inverse and linear (Fig. 61). However, there was little difference in the LD_{50} values of the virus for chicks of ages 7 and 10 hours (Table 35).

The undiluted inoculum did not induce a clinical state in chickens of 9 and 35 days of age when injected subcutaneously.

Effect of Dose: The dose of virus used to infect chickens also influenced the mortality pattern in experimental Aujeszky's infection induced by intracerebral, intramuscular or subcutaneous inoculation. Evidence in this regard first stemmed from the results of the experiment in which chicks of different ages were

injected intracerebrally (Table 30). In general, higher infective doses induced greater percentages of deaths and the relationship between dose and mortality in each age group was direct and linear (Fig. 62), and the values in 4 of the 5 groups were highly significant (Table 36). Moreover, comparison of the regressions of mortalities on dose in respect of chickens of 1, 14, 21, 28 and 45 days of age revealed that the slopes of the regressions were similar ($F = 2.61$, d.f. 4, 25) but the levels were different ($F = 27.00^{**}$, d.f. 4, 25). Confirmation of the finding that mortality in chickens injected with Aujeszky's virus was linearly related to the size of inoculum emerged from the results of other studies in which chicks of ages ranging from a few to 24 hours were infected intramuscularly or subctuaneously (Tables 33 and 35; Figs. 63 and 64). The relationship between the percentage of deaths and the dose of virus was direct, linear and significant both in the subcutaneously ($F = 29.53^{**}$, d.f. 1, 16) and intramuscularly infected groups of chicks ($F = 9.78^{*}$, d.f. 1, 10). Furthermore, comparison of the two regressions of mortality on dose revealed that the slopes were identical ($F = 0.43$, d.f. 1, 26) although the levels were different ($F = 7.71^{**}$, d.f. 1, 25). In chicks infected subcutaneously, the duration of fatal infection was also linearly

and significantly related to the dose of virus ($\hat{Y}$ = 6.14 - 0.41.X; r = 0.376*, where X is the dose of virus, $\hat{Y}$ is the line of best fit and r the correlation coefficient). Higher doses generally induced deaths earlier than lower doses (Table 37).

THE WEYBRIDGE AND THE HUNGARIAN STRAINS

The Weybridge Strain: In a sighting experiment, a 10 per cent suspension of the infective brain was injected into two groups of day-old chicks, one intramuscularly and the other intracerebrally in 0.2 and 0.05 ml. amounts respectively. The infectivity titre of the suspension for PK15 cells ranged from $10^{4-4.2}$ $TCID_{50}$ per gram of tissue. The results (Table 38) showed that chicks of this age group were highly susceptible to experimental infection with Aujeszky's virus; there were no survivors. Between the two groups of experimental chicks, there were significant differences in the mean duration of infection ($t = 2.677^{*}$; $P < 0.05$) and in the amounts of virus present in the brain ($t = 4.440^{**}$; $P < 0.01$) suggesting that the intracerebral route was more effective than the intramuscular route.

In another experiment, groups of chickens of different ages were infected in 0.05 ml. amounts intracerebrally with the Weybridge Strain which had been

passaged serially in PK15 cells and which had a titre of $10^{8.2}$ $TCID_{50}$ per ml. in the 3rd passage. The mortalities in infected chickens varied according to age (Table 39) and the relationship between age and deaths was inverse, linear and significant (Fig. 65). The results also showed that the influence of the dose of virus was more definitive on the mean duration of infection (Table 40) than on the percentage of deaths. The onset of deaths occurred earlier in chickens infected with the higher doses of the virus than in those receiving lower doses, and the relationship between the size of inoculum and time of death in the infected birds was direct, linear and significant ($\hat{Y} = 7.76 - 0.73.X$; $r = 0.674^{**}$ where X is the dose of virus injected, $\hat{Y}$ is the regression between dose and time of death and r the correlation coefficient). On the other hand, the influence of the dose of the virus on the percentage of deaths was not clear cut, a significant linear relationship being evident in one group of chicks only (Table 41).

The Hungarian Strain: This strain had an infectivity titre of $10^{7.5}$ $TCID_{50}$ per ml. for PK15 cells and when inoculated intracerebrally induced a clinical state followed by death in day-old chicks (Table 42), whereas two other groups of chickens aged 10 and 84 days

respectively responded uneventfully after being infected with different dilutions of the same virus pool. Intramuscular infection was also effective in establishing overt infection in very young chicks and the influence of the age of the host on mortalities was evident in the LD_{50} values of the virus for 1 and 2 days old chicks (Table 43), the difference being highly significant ($t = 3.723^{**}$; $P < 0.01$).

Analysis of data of mortalities in day-old chicks infected by the two routes revealed similar features. For instance, the effect of the dose of virus on the percentage of mortalities in the two routes of infection was direct, linear and significant (Fig. 66) and the two regressions were identical in slopes as well as levels ($F = 0.37$, d.f. 1, 12; $F = 1.59$, d.f. 1, 12). Moreover, the mean periods of infection in the intracerebrally and intramuscularly infected chicks were inversely related to the dose (Tables 42 and 44) and the lines of best fit were linear and significant ($F = 22.17^{**}$, d.f. 1, 37 and $F = 16, 80^{**}$, d.f. 1, 73 respectively; Fig. 67). However, the two regressions differed markedly in slopes and levels.

That the effect of dose on mortalities was independent of that of age was clear when the regressions of mortalities on dose in intramuscularly infected 1 and

2 days old chicks were compared. The data revealed that the slopes of the two regression lines were identical ($F = 7.30^{*}$, d.f. 1, 12; Fig. 68).

It was interesting that the LD_{50} value of the virus for intramuscularly infected day-old chicks was significantly higher than that for intracerebrally infected chicks of the same age ($t = 2.431^{*}$; $P < 0.05$).

VIRUS DISTRIBUTION

The McFerran Strain : Intracerebral infection: The virus was recovered from the brain tissues of all chickens aged 1 to 180 days dying of experimental intracerebral infection. Only once was the virus isolated from a specimen of liver and on no occasion was it detected in the heart, lung, spleen, spinal cord and kidney. Chickens of different ages that did not respond clinically to intracerebral infection with the virus were killed at different intervals post-infection from the 10th day onwards. Pooled brain suspensions from these birds were inoculated into cell cultures but none showed evidence of virus activity.

In an attempt to demonstrate the presence of virus in the blood, groups of chickens of different ages were killed at intervals ranging from 5 minutes to 72 hours post-infection and pooled specimens of blood were seeded into PK15 cells. The results of this experiment showed

that the rates of virus recovery from blood were inversely related to the age at which the chickens were infected (Table 45). In infected day-old chicks, virus was detected in the blood up to 66 hours post-infection and the mean duration of viraemia was 16 ± 5.5 hours. On the other hand, the estimated mean periods of viraemia for infected chicks of 7 and 28 days of age were 2.3 ± 1.6 and 3 ± 1.3 hours respectively. Limited quantitative studies regarding the amount of virus present in the blood of 1 and 7 days old birds showed that the titres declined rapidly (Table 46). The virus was not recovered from the blood of birds aged 7 weeks or older; nor was it detected in the blood of day-old chicks that were inoculated with only 1,000 $TCID_{50}$ of virus.

Virus titres in the brains of chicken dying of intracerebral infection ranged from 1.7 to 5.5 $TCID_{50}$ per gram. Whereas a significant inverse linear relationship was detected between the mean virus titres in the brains of chicks that died and the time of death ($\hat{Y} = 4.34 - 0.12.X$; $r = 0.526^{**}$, where X is the day of death and $\hat{Y}$ is regression of titres on time, and r the correlation coefficient, Table 47), the titres were not influenced either by the age at which the birds were infected ($F = 0.91$, d.f. 16, 21) or by the dose of virus they had received ($F = 1.54$, d.f. 1, 21; Table 48). On the other

hand, the rate of replication of the virus in the brains of infected chicks seemed to be related to the age at which the birds were infected (Table 49, Fig. 69). In day-old birds killed at different intervals following intracerebral infection with a high dose of virus, evidence of virus replication in the brain was manifest at 4 hours; but the latent period was longer in birds of 14 - 28 days of age. In both groups of chickens the rise in virus titres was exponential, and peak titres were reached at 24 and 48 hours respectively. However, in young chicks infected intracerebrally with only 1,000 $TCID_{50}$ doses of the virus, replication of the virus in the brain tissue was first evident at 16 hours (Table 50).

Intramuscular infection: Virus was present in the brain and in the muscle tissue at the site of inoculation of all chicks that died following intramuscular infection. In many cases virus was also detected in the spinal cord. On the other hand, virus was rarely recovered from the kidney, heart and lung.

As with intracerebral infection, the mean duration of viraemia in intramuscularly infected birds was related to the age at which they were infected (Table 51), the relationship being inverse, linear and significant ($\hat{Y} = 18.93 - 3.27.X$; $r = -0.434^{**}$, where X is the age of the host, $\hat{Y}$ is the regression line and r the correlation

coefficient). The mean viraemic periods in the infected 1, 2 - 3 and 7 days-old chicks were 12.8 ± 2.54, 11.0 ± 3.39 and 1.3 ± 0.21 hours, respectively.

Following intramuscular infection, the mean virus titres in the muscle tissue at the sites of inoculation, brain and spinal cord differed little in chickens of different age groups that were inoculated with various doses of the virus ($F = 0.11$, d.f. 2, 39 and $F = 0.61$, d.f. 7, 39 respectively; Table 52). In experiments designed to study the replication of the virus in the tissues of intramuscularly infected chickens, it was observed that the amounts of virus recovered from the muscle tissue at the sites of inoculation decreased with time (Table 53). There was evidence also of replication of the virus between 2 and 4 hours post-infection, although the titres remained largely unchanged in the experimental period of 5 days (Fig. 70). Viraemia was evident at 2 hours, but the titres were low and the virus was not detected after 24 hours. Virus was detected in the spinal cord 18 hours post-infection but it was demonstrable in the brain only on the second day. The data relating to recovery of the virus from chicks infected intramuscularly at 18 to 42 hours of age were similar to those obtained from the younger infected chicks; but in chicks infected at 3 weeks of age the

virus was not recovered from the site of inoculation after 48 hours, nor was there evidence of replication of the virus in the brain or spinal cord. Virus was not detectable in pharyngeal and cloacal swabs collected daily for a period of 10 days post-infection from 10 chicks of $<$ 24 hours of age that were infected intramuscularly with undiluted virus ($10^{7.5}$ $TCID_{50}$/per ml.)

The Hungarian Strain: Inoculation of chicks less than 24 hours of age with the Hungarian strain resulted in the virus being distributed in the heart, lung, liver, spleen and kidney, in addition to the brain and spinal cord (Table 54). In chickens infected by the intramuscular route, the virus titres in the muscle tissue at the site of inoculation were consistently high but, in general, there were no differences in the titres in the various tissues between intramuscularly and intracerebrally infected chicks. Chicks infected intramuscularly with a higher dose of virus had greater amounts of virus in their tissues than those infected with a lower dose; the difference being significant ($t = 2.159^{*}$; $P < 0.05$).

In day-old chicks, invasion of the bloodstream occurred earlier following intracerebral inoculation than intramuscular inoculation but in both groups of chicks the viraemia persisted until 48 hours post-infection

(Table 55). In older chickens, however, there was no difference either in the onset or duration of the viraemia.

VIRUS-SPECIFIC TISSUE ANTIGENS

Virus-specific, complement-fixing antigens were not detected in suspensions of heart, lung, spleen, liver, kidney and brain from young chicks infected intramuscularly or intracerebrally with the McFerran strain of Aujeszky's virus. But, when different aliquots of brain suspensions were pooled and concentrated approximately 50 times by treatment with ammonium sulphate, specific CF antigens were detected, and the preparation had a titre of 2.5 $\log_{10}$ per ml. On the other hand, the tissues of chicks infected intramuscularly or intracerebrally with the Hungarian strain of virus contained specific CF antigens with titres ranging from 2 to 3.2 $\log_{10}$ units per ml. (Table 56). Antigens were not detected in the spleen and liver. No relationship existed between the antigen titres in the tissues and the routes of infection or the dose of virus inoculated; nor was there any relationship between the virus and antigen titres in the tissues tested (Tables 54 and 56).

Furthermore, specific immunodiffusion antigens were not detected in the tissues of chicks infected either with the McFerran or the Hungarian strains of Aujeszky's

virus.

RESISTANCE TO REINFECTION

The results of the present studies on the pathogenicity of the McFerran strain of Aujeszky's virus for chickens, as presented in the foregoing sections, show that there is a gradual extension of the mean survival period and a reduction in the percentage of mortality in infected chickens of the higher age groups. The nature of the age-associated resistance was further investigated by challenging those groups of chickens that had survived the primary infection by inoculating them intracerebrally with $10^{6.2}$ $TCID_{50}$ of virus. A number of control birds of similar ages were also infected. Analysis of the data from a pilot experiment (Table 57) in which chicks of different ages were first infected intramuscularly and then challenged intracerebrally revealed that the ability of the chicks to resist a high dose of virus was largely influenced by the amount of virus in the primary inoculation. In general, chickens that received up to $10^{2.8}$ $TCID_{50}$ of virus in the primary inoculum withstood the challenge carried out 14 to 28 days later, whereas a large number of control chickens of corresponding ages succumbed to the infection. It was also clear that the percentage of survivors from the challenge infection was not influenced by the age at

which the chicks were first infected (F = 0.12, d.f. 1, 40). On the other hand, in all but one of the age groups of intramuscularly infected chickens, the percentage of recoveries from intracerebral challenge was directly related to the amount of virus they had received in the primary inoculation; and the relationships were linear and significant (Table 58). The preliminary evidence that resistance to reinfection was significantly influenced by the degree of previous exposure to the virus was confirmed in other experiments in which chickens of different ages were infected intracerebrally or subcutaneously and then challenged intracerebrally after periods ranging from 14 to 32 days. A significant number of control chickens of corresponding ages was included in each challenge infection.

Analysis of the results indicated that in chicks first exposed to the virus subcutaneously or intracerebrally, the ability to resist intracerebral challenge infection was not conditioned by the age of the bird (F = 0.03, d.f. 1, 14 and F = 0.10, d.f. 1, 46 respectively) but was related to the amount of virus contained in the primary inocula (Tables 59 and 60). In most age groups where the chickens were first exposed to the virus subcutaneously or intracerebrally, the percentage of recoveries from challenge infection was directly and

significantly influenced by the dose of virus in the primary inocula and the lines of best fit were linear (Tables 61 and 62). The fact that a large number of control chickens of corresponding ages succumbed in each challenge infection also suggested that recovery from challenge in the infected groups was not due to age-associated tolerance.

In a further investigation that was carried out to determine the onset of resistance to reinfection, groups of chicks previously inoculated with the virus intramuscularly were challenged intracerebrally on days 1, 4, 8, 11 and 14 post-infection together with a large number of control chicks of corresponding ages. The results of this experiment (Table 63) showed that specific resistance to reinfection had developed as early as the 4th day after primary infection, and a comparison of the responses of infected and control groups of chicks on days 1 and 8 after challenge suggested that the high percentage of recoveries from reinfection in the first group of chicks were possibly not due to age-associated tolerance.

NEUTRALISING ANTIBODIES

Preliminary tests with sera from chickens that recovered from a single exposure to the McFerran strain of Aujeszky's virus showed that the titres of neutralising

antibodies ranged from <1.3 to 1.9 $\log_{10}$ units per ml. On the other hand, in chickens that survived infection with more than one dose of live virus administered parenterally at intervals of 7 days or more, the titres of neutralising antibodies that developed were relatively higher (Table 64), but there was no correlation between neutralising antibody titres and the number of inoculations the chickens received ($F = 1.08$, d.f. 2, 34), nor was there a correlation between the antibody titres and the time of sampling ($F = 1.91$, d.f. 1,12).

In a sighting experiment it was observed that when chickens were inoculated intravenously or subcutaneously with two doses of $10^{7.50}$ $TCID_{50}$ of virus which had been inactivated at 56°C for 60 minutes, neutralising antibodies were not detectable in their sera (Table 65). Similarly, in two other groups of chickens that were infected intravenously or subcutaneously with 2 doses of live virus from the same virus pool, neutralising antibodies were either not detected or occurred in low levels. The tests were carried out with pooled sera from 3 chickens. When the 2 groups of chickens sensitized with inactivated virus were injected intravenously 16 days later with $10^{7.5}$ $TCID_{50}$ of live virus, neutralising antibodies developed, the titres ranging

from 2.0 to 2.5 $\log_{10}$ units per ml. In one of the 2 groups of chicks first exposed to live virus, an anamnestic antibody response of a low order developed when they were re-inoculated with inactivated virus.

The experiment was repeated using live and inactivated suspensions of the McFerran and the Hungarian strains of virus (Tables 66 and 67). The titres of the virus suspensions were $10^{7.5}$ $TCID_{50}$ and $10^{7.0}$ $TCID_{50}$ respectively when titrated in PK15 cells and the $TCID_{50}$ dose used to infect the chickens was log $10^{6.0}$. In birds infected with suspensions of live virus, a low level of neutralising antibodies was detected and the titres increased marginally in two of the three groups when a second dose of live virus was given (Table 67). On the other hand, inactivated suspensions of the 2 virus strains did not elicit antibody responses even after the second inoculation. When challenged on the 16th day by intracerebral inoculation of live virus, unequivocal anamnestic antibody responses occurred only in those groups that were first exposed to live virus; the corresponding antibody titres in chickens sensitized with inactivated virus were relatively low (Table 67).

Comparison of the antibody responses to intracerebral challenge in the four groups of chickens showed that there was no apparent difference in the sensitization induced by one or two doses of either live or

inactivated virus suspensions (Tables 66 and 67). Also no significant differences were noted in the antibody titres within the 2 groups of chickens first exposed to either the Hungarian or the McFerran strain of live virus (Table 68). Similarly, there were no significant differences in the antibody titres in the 2 groups of chickens first inoculated with 2 doses of either the Hungarian or McFerran strain of inactivated virus (Table 68). On the other hand, the mean antibody titres in chickens sensitized with the live McFerran strain were significantly higher than the titres induced by the homologous inactivated virus. Likewise, the live Hungarian strain elicited antibody responses of a significantly higher magnitude than did the inactivated suspension.

An experiment was designed with the objective of tracing the sequential development of neutralising antibodies in chickens. It was observed that following the intracerebral inoculation of 2 doses of $10^{6.9}$ $TCID_{50}$ of the live McFerran strain of Aujeszky's virus, neutralising antibody titres developed only after the second inoculation and were maintained at low levels. However, a marked anamnestic increase in the antibody titres occurred when 4 of the chickens were inoculated intravenously with a third dose of live virus at 8 weeks

post-infection (Table 69). Low levels of neutralising antibodies persisted up to 560 days in four birds. When challenged intravenously with live virus, a recall response occurred in the birds accompanied by the production of high levels of neutralising antibodies (Table 70).

Incorporation of guinea pig complement in the serum-virus mixtures was of no advantage in the virus neutralization tests using early and late chicken convalescent sera (Table 71). No true differences in titres occurred when parallel tests were carried out with and without added complement. Nor did the results of preliminary studies reveal differences in the neutralising antibody titres when convalescent chicken sera, which were stored at -20°C, were used unheated or after inactivation at 56°C for 30 minutes.

TABLE 28

INFLUENCE OF THE ROUTE OF INOCULATION OF $10^{5.7}$ $TC1D_{50}$ OF THE McFERRAN STRAIN OF AUJESZKY'S VIRUS ON THE MORTALITY PATTERN IN CHICKS LESS THAN 24 HOURS OF AGE

Route	Responses	Percentage of Mortality	Mean death time (days) + S.E.
Intracerebral	10/10	100	1.90 ± 0.43
Intramuscular	12/15	80	3.33 ± 0.28
Intradermal	8/11	73	5.25 ± 0.59
Subcutaneous	9/15	60	3.20 ± 0.36
Intranasal	7/27	26	7.43 ± 0.81
Intraocular	5/27	19	7.00 ± 1.26

Numerator = number dead Denominator = number inoculated

TABLE 29

INFLUENCE OF AGE ON THE MORTALITY PATTERN IN CHICKENS INFECTED INTRACEREBRALLY WITH THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS*

Age (Days)	Responses	Percentage of deaths	Time of death (days)								
			1	2	3	4	5	6	7	8	9
1	20/20	100	8	6	3	1	2	-	-	-	-
7	15/17	88	5	7	2	-	-	-	-	-	-
14	15/19	79	-	4	3	1	6	1	-	-	-
21	15/17	88	1	3	-	2	4	4	-	-	1
28	21/26	81	2	-	-	4	12	2	-	1	-
42-49	17/24	71	1	-	3	2	6	2	2	1	-
90-180	6/8	75	-	-	-	3	-	2	-	1	-

*$TC1D_{50}$ of virus for PK15 cells was $10^{7.5}$ per ml.

Numerator = number dead Denominator = number inoculated

TABLE 30

INFLUENCE OF THE DOSE OF THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS* ON THE MORTALITY PATTERN IN CHICKENS OF DIFFERENT AGES INFECTED INTRACEREBRALLY

Age (days)		Log dose ($TC1D_{50}$/0.1 ml.)								LD_{50} + S.E.
		6.5	5.5	4.5	3.5	2.5	1.5	0.5	0.05	
1	Responses	10/10	10/10	9/10	9/10	8/10	4/10	7/10	0/10	$10^{-5.20} \pm 0.31$
	Percentage of deaths	100	100	90	90	80	40	70	0	
7	Responses	5/5	3/5	4/5	3/5	3/5	3/5	0/5	-	$10^{-3.70} \pm 0.53$
	Percentage of deaths	100	60	80	60	60	60	60	0	
14	Responses	7/8	5/8	7/8	3/8	2/8	2/8	0/8	0/8	$10^{-2.75} \pm 0.39$
	Percentage of deaths	88	63	88	38	25	25	0	0	
21	Responses	6/7	7/8	5/10	3/5	1/5	0/5	-	-	$10^{-2.53} \pm 0.40$
	Percentage of deaths	86	88	50	60	20	0			
28	Responses	9/13	12/13	10/15	7/10	3/10	2/10	0/5	0/5	$10^{-2.98} \pm 0.51$
	Percentage of deaths	69	92	67	70	30	20	0	0	
42-49	Responses	6/10	4/7	4/8	1/8	1/8	1/7	0/5	0/5	$10^{-1.56} \pm 0.39$
	Percentage of deaths	60	57	50	13	13	14	0	0	

* The infectivity titre for PK15 cells was $10^{7.5}$ per ml.

Numerator = number dead Denominator = number inoculated.

TABLE 31

COMPARISONS OF LD_{50} VALUES OF THE McFERRAN STRAIN OF AUJESZKY'S VIRUS IN CHICKENS OF DIFFERENT AGES INFECTED INTRACEREBRALLY

Ages (days)	LD_{50}	Difference	S.E. of difference	d.f.	t	P
1 and 7	$_{10}^{-5.20 + 3.70}$	1.500	0.574	13	2.61*	<0.05
7 and 14	$_{10}^{-3.70 + 2.75}$	0.095	0.645	11	1.47	>0.05
7 and 21	$_{10}^{-3.70 + 2.53}$	1.170	0.660	11	1.77	>0.05
7 and 45	$_{10}^{-3.70 + 1.56}$	2.140	0.649	11	3.30**	<0.01

TABLE 32

THE INFLUENCE OF AGE ON THE MORTALITY PATTERN IN CHICKENS INFECTED INTRAMUSCULARLY WITH THE McFERRAN STRAIN OF AUJESZKY'S VIRUS

Log dose ($TCID_{50}$)	Median age	No.dead / No.infected	Percentage of deaths	Day of death									Mean time of death (days) + S.E.
				1	2	3	4	5	6	7	8	9	
6.0	4 hours	9/9	100			5	3					1	4.0 ± 0.68
6.0	7 "	11/11	100		2	9							2.8 ± 0.13
6.0	10 "	6/7	86			3	2	1					3.7 ± 0.37
6.0	13 "	21/30	70	1		7	8		2			3	4.4 ± 0.49
6.0	37 "	4/15	27			2	1	1					3.8 ± 0.48
6.0	57 hours	0/3	0										
6.0	3 days	0/18	0										
6.0	6 "	0/20	0										
6.3	8 "	0/4	0										
6.7	21 "	0/5	0										
6.7	42 "	0/5	0										

TABLE 33

INFLUENCE OF THE DOSE OF THE McFERRAN STRAIN OF AUJESZKY'S VIRUS* ON THE MORTALITY PATTERN IN CHICKENS OF DIFFERENT AGES INFECTED INTRAMUSCULARLY

Median age (hours)		Log dose of virus ($TCID_{50}$/0.1 ml.)							
		6.0	5.0	4.0	3.0	2.0	1.0	0.1	LD_{50} + S.E.
7	Responses	20/20	12/14	9/14	4/8	0/8	0/7	0/3	$10^{-2.39\pm0.30}$
	Percentage of deaths	100	86	64	50	0	0	0	
10	Responses	6/7	7/8	6/8	1/8	0/6	0/6	0/3	$10^{-2.25\pm0.36}$
	Percentage of deaths	86	88	75	13	0	0	0	
13	Responses	21/30	5/21	4/21	1/15	0/13	0/10	0/10	$10^{-0.57\pm0.21}$
	Percentage of deaths	70	24	19	7	0	0	0	
37	Responses	4/15	0/7	3/7	1/7	0/7	0/5	0/5	
	Percentage of deaths	27	0	43	14	0	0	0	
57	Responses	0/3	0/3	0/3	0/3	0/3	0/3	-	
	Percentage of deaths	0	0	0	0	0	0	0	
72	Responses	0/4	0/4	0/4	0/4	0/4	0/4	0/4	
	Percentage of deaths	0	0	0	0	0	0	0	

*Infectivity titre for PK15 cells was $10^{7.0}$ per ml.

Numerator = number dead Denominator = number inoculated - = not done

TABLE 34

COMPARISON OF LD_{50} VALUES OF THE McFERRAN STRAIN OF AUJESZKY'S VIRUS FOR INTRAMUSCULARLY INFECTED CHICKS OF DIFFERENT AGES

Age (hours)	Difference in LD_{50} values	S.E. of the difference	d.f.	t	p
7 and 10	$10^{-0.14}$	0.474	17	0.295	>0.50
7 and 13	$10^{-1.82}$	0.348	28	5.259**	<0.010
10 and 13	$10^{-1.68}$	0.400	23	4.200**	<0.010

TABLE 35

INFLUENCE OF THE DOSE OF THE McFERRAN STRAIN OF AUJESZKY'S VIRUS* ON THE MORTALITY PATTERN IN CHICKS OF DIFFERENT AGES INFECTED SUBCUTANEOUSLY

Median age (hours)		Log dose of virus ($TC1D_{50}$/0.2 ml.)								
		6.3	5.3	4.3	3.3	2.3	1.3	0.3	0.03	LD_{50} + S.E.
10	Responses	7/8	4/4	3/4	0/4	1/4	2/4	0/4	0/4	$10^{-2.60 \pm 0.63}$
	Percentage of deaths	88	100	75	0	25	50	0	0	
13	Responses	7/9	6/8	5/8	4/8	1/8	1/8	0/8	0/8	$10^{-2.42 \pm 0.45}$
	Percentage of deaths	78	75	63	50	13	13	0	0	
21	Responses	4/7	1/2	0/2	0/2	0/2	0/2	0/2	0/2	
	Percentage of deaths	57	50	0	0	0	0	0	0	
34	Responses	3/8	0/3	0/3	0/3	0/3	0/3	0/3	0/3	
	Percentage of deaths	38	0	0	0	0	0	0	0	
60	Responses	1/8	0/5	1/5	0/5	0/5	0/5	0/5	0/5	
	Percentage of deaths	0	0	0	0	0	0	0	0	

*The virus had an infectivity titre of $10^{7.0}$ $TC1D_{50}$/ml for PK15 cells

Numerator = number dead Denominator = number inoculated

TABLE 36

REGRESSIONS OF THE PERCENTAGE OF MORTALITY IN CHICKENS OF DIFFERENT AGES ON THE DOSE OF THE McFERRAN STRAIN OF AUJESZKY'S VIRUS INOCULATED

Age (days)	Regression	Degrees of freedom	Correlation coefficient
1	$\hat{Y} = 7.86.X + 53.92$	5	0.802*
7	$\hat{Y} = 8.21.X + 31.26$	5	0.585
14	$\hat{Y} = 14.39.X - 3.60$	5	0.943**
21	$\hat{Y} = 16.57.X - 14.57$	5	0.955**
28	$\hat{Y} = 13.86.X - 1.21$	5	0.902**
45	$\hat{Y} = 10.82.X - 8.30$	5	0.938**

TABLE 37

THE RELATIONSHIP BETWEEN THE DOSE OF THE McFERRAN STRAIN OF AUJESZKY'S VIRUS* AND THE MEAN DAY OF DEATH IN CHICKS LESS THAN 18 HOURS OLD INFECTED SUBCUTANEOUSLY.

Log dose of virus ($TCID_{50}$/0.2 ml.)	Responses	Day of death								Mean day of death + S.E.
		2	3	4	5	6	7	8	9	
6.3	14/17	1	9	3		1				3.4 ± 0.3
5.3	10/12		5	4		1				3.70 ± 1.0
4.3	8/12		1	3		2			2	5.6 ± 1.0
3.3	4/8			2	2					4.5 ± 0.3
2.3	2/12		1				1			5.0 ± 2.0
1.3	3/12	1		1				1		4.7 ± 1.8
0.3	0/12									

*The infectivity titre of the virus for PK15 cells was $10^{7.0}$ $TCID_{50}$ per ml.

Numerator = number dead Denominator = number inoculated

TABLE 38

RESPONSES OF YOUNG CHICKS TO EXPERIMENTAL INFECTION

WITH THE WEYBRIDGE STRAIN OF AUJESZKY'S VIRUS

Parameter	Route of inoculation	
	Intramuscular ($10^{3.5}$ $TCID_{50}$/gm.)	Intracerebral ($10^{2.9}$ $TCID_{50}$/gm.)
No. dead/ No. infected	4/4	6/6
Mean duration of fatal infection (days) + S.E.	4 ± 0.25	2.2 ± 0.60
Virus titres in brain + S.E.	3.02 ± 0.41 3.53 ± 0.39	5.20 ± 0.28 5.33 ± 0.31

TABLE 39

MORALITY PATTERN IN CHICKENS OF DIFFERENT AGES INFECTED INTRACEREBRALLY WITH THE WEYBRIDGE STRAIN OF AUJESZKY'S VIRUS*

Log dose of virus ($TCID_{50}$/0.05 ml.)	Age (days)							
	2		21		28		49	
	Responses	percentage of deaths	Responses	percentage of deaths	Responses	percentage of deaths	Responses	percentage of deaths
6.9	3/3	100	2/3	67	3/3	100	1/5	20
5.9	4/4	100	1/3	33	1/3	33	0/4	0
4.9	5/5	100	1/3	33	1/3	33	1/5	20
3.9	7/7	100	0/3	0	0/3	0	0/5	0
2.9	7/7	100	0/3	0	0/3	0	0/5	0
1.9	7/7	100	0/6	0	0/3	0	0/5	0
0.9	4/7	57	0/4	0	0/3	0	0/5	0
0.09	0/6	0	0/4	0	0/5	0	0/5	0

*The infectivity titre of the virus for PK15 cells was $10^{8.2}$ per ml.

Numerator = number dead Denominator = number inoculated

TABLE 40

DISTRIBUTION OF MORTALITIES IN TWO DAYS OLD CHICKS INTRACEREBRALLY INFECTED WITH THE WEYBRIDGE STRAIN OF AUJESZKY'S VIRUS*

Log dose of virus ($TCID_{50}$/0.05 ml.)	Responses	Day of death										Mean day of death + S.E.
		1	2	3	4	5	6	7	8	9	10	
6.9	3/3		3									2.00 ± 0.00
5.9	4/4			3	1							3.30 ± 0.27
4.9	5/5				3	2						4.40 ± 0.24
3.9	7/7			2		2	3					4.86 ± 0.51
2.9	7/7	1			1	1	2	2				5.14 ± 0.80
1.9	7/7					3	1	1		2		6.57 ± 0.68
0.9	4/7					1			1		2	8.25 ± 1.20

*The virus had an infectivity titre of $10^{8.2}$ $TCID_{50}$ per ml. for PK15 cells

Numerator = number dead. Denominator = number inoculated.

TABLE 41

COMPARISON OF THE REGRESSIONS OF THE PERCENTAGES OF MORTALITIES ON DOSE IN CHICKENS OF DIFFERENT AGES INOCULATED INTRACEREBRALLY WITH THE WEYBRIDGE STRAIN OF AUJESZKY'S VIRUS

Age (days)	Regression	d.f.	Correlation coefficient
1	$\hat{Y} = 4.60.X + 75.92$	5	0.613
21	$\hat{Y} = 15.56.X - 49.84$	3	0.882*
28	$\hat{Y} = 23.30.X - 80.97$	3	0.825
49	$\hat{Y} = 4.00.X - 11.60$	3	0.577

TABLE 42

MORTALITY PATTERN IN DAY-OLD CHICKS INFECTED INTRACEREBRALLY WITH THE HUNGARIAN STRAIN OF AUJESZKY'S VIRUS*

Log dose of the virus ($TCID_{50}$/0.05 ml.)	Responses	Percentage of deaths	Day of death									Mean time of death (days) + S.E.
			1	2	3	4	5	6	7	8	9	
6.2	5/5	100	-	1	2	-	1	1	-	-	-	3.8 ± 0.74
5.2	8/9	89	-	-	1	2	1	2	2	-	-	5.3 ± 0.53
4.2	7/9	78	-	-	-	1	2	2	2	-	-	5.7 ± 0.42
3.2	7/9	78	-	-	-	1	1	3	2	-	-	5.9 ± 0.41
2.2	6/9	67	-	-	-	-	1	2	2	1	-	6.5 ± 0.43
1.2	5/9	46	-	-	-	-	-	-	3	2	-	5.4 ± 0.25
0.2	1/3	33	-	-	-	-	-	1	-	-	-	6.0 ± 0.00
0.02	0/3	0	-	-	-	-	-	-	-	-	-	
LD_{50}	$10^{-4.37} \pm 0.45$											

*The virus had an infectivity titre of $10^{7.5}$ $TCID_{50}$ per ml for PK15 cells

Numerator = number died Denominator = number inoculated - = no death

TABLE 43

MORTALITY PATTERN IN YOUNG CHICKS INFECTED INTRAMUSCULARLY WITH THE HUNGARIAN STRAIN OF AUJESZKY'S VIRUS

Log dose of the virus ($TCID_{50}$/0.2 ml.)	Age			
	Day-old		Two-days old	
	Responses	Percentage of deaths	Responses	Percentage of deaths
6.8	6/6	100	5/5	100
5.8	14/15	93	4/5	80
4.8	8/8	100	5/5	100
3.8	9/9	100	4/5	80
2.8	9/9	100	1/5	20
1.8	7/8	88	1/5	20
0.8	2/3	67	0/5	0
0.08	0/3	0	0/5	0
LD_{50}	$10^{-5.76} \pm 0.35$		$10^{-3.5} \pm 0.40$	

Numerator = number dead Denominator = number infected

TABLE 44

DISTRIBUTION OF MORTALITIES IN YOUNG CHICKS INFECTED INTRAMUSCULARLY WITH DIFFERENT DOSES OF THE HUNGARIAN STRAIN OF AUJESZKY'S VIRUS

Log dose of virus ($TCID_{50}$/0.2 ml.)	No. dead / No. infected	Day of death						Mean time of death (days)±S.E.
		1	2	3	4	5	6	
6.8	11/11	-	3	4	2	2	-	3.3 ± 0.32
5.8	18/20	1	5	8	4	-	-	2.8 ± 0.20
4.8	13/13	-	1	9	2	1	-	4.0 ± 0.20
3.8	13/14	-	-	6	5	2	-	3.7 ± 0.21
2.8	10/14	-	-	4	4	2	-	3.8 ± 0.25
1.8	8/13	-	-	3	4	-	1	3.9 ± 0.35
0.8	2/7	-	-	-	-	1	1	5.5 ± 0.5
0.008	0/8	-	-	-	-	-	-	0

TABLE 45

VIRAEMIA IN CHICKENS INFECTED INTRACEREBRALLY WITH $10^{5.7}TCID_{50}$ OF THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Period of Post-infection (hours)	Age of chickens (days)		
	1	7	28
1	3/3	5/6	1/1
2	1/2	3/4	1/1
4	1/2	2/3	0/2
6	1/3	1/5	1/2
12	3/4	0/1	0/2
18	1/1		
24	1/2		
48	1/5		
66	1/1		
72	0/1		

Numerator = number positive Denominator = number of samples tested

TABLE 46

VIRUS TITRES* IN THE BLOOD OF CHICKENS INFECTED INTRACEREBRALLY WITH $10^{5.7}$ $TC1D_{50}$ OF THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Period Post-infection (hours)	Age of Chickens (days)	
	1	7
0.08		3.5
0.25	3.0	3.3
0.50	2.5	3.3
1		2.8
2		2.8
3		1.5
4		1.0
6		<1.0
12	2.0	

*Expressed as the negative logarithm of number of cell culture infective units per ml.

TABLE 47

MEAN VIRUS TITRES IN THE BRAINS OF CHICKENS INFECTED INTRACEREBRALLY WITH THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS IN RELATION TO DURATION OF INFECTION

Day of Death	Number of specimens titrated	Titre + S.E.
1	4	5.00 ± 0.63
2	6	3.91 ± 0.39
3	6	3.73 ± 0.31
4	6	3.61 ± 0.39
5	14	3.70 ± 0.29
6	11	3.57 ± 0.42
7	2	3.20 ± 0.00
8	10	3.45 ± 0.21
9	5	3.70 ± 0.75

TABLE 48

MEAN VIRUS TITRES* IN THE BRAINS OF CHICKENS OF DIFFERENT AGES INFECTED INTRACEREBRALLY WITH THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Dose of Virus (TC^1D_{50}/0.05 ml.)	Age of chickens (days)					
	1	7	14	21	28	45
6.2	4.63	4.35	2.70	4.20	4.20	2.85
5.2	4.38	4.20	3.20	4.70	3.20	2.70
4.2	3.70	1.70	4.20	3.37	4.37	3.20
3.2	4.20	4.70	3.20	3.20	3.20	3.20
2.2	4.53	1.70	1.70	3.20	3.20	3.20
1.2	2.82	4.20	1.70	1.70	1.70	1.70
0.2	4.32	1.70	1.70	ND	ND	ND

*Expressed as the negative logarithm of cell culture infective units per ml.

ND = not done

TABLE 49

DEVELOPMENT OF VIRUS TITRES* IN THE BRAINS OF CHICKENS INFECTED INTRACEREBRALLY WITH $10^{6.2}$ $TC1D_{50}$ OF THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Hours post infection	Age (days)		
	1	14-28	36
0	2.0	2.3	2.5
0.5	<1.0		
1	<1.0	2.0	
2	<1.0	1.7	
4	1.0	<1.0	<1.0
6	1.0	<1.0	
8	2.3	1.0	
12	3.6	1.5	<1.0
18	4.7	1.5	
24	5.0	2.0	2.5
30	5.0		
48	4.4	4.0	2.5
72		3.5	
96	3.5	3.5	3.0

TABLE 50

DEVELOPMENT OF VIRUS TITRES IN THE BRAINS OF 1 TO 2 DAY OLD CHICKS INFECTED INTRACEREBRALLY WITH 1000 $TC1D_{50}$ OF THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Period post infection (hours)	Clinical signs	Titre ($log_{10}TC1D_{50}$/gm)
0.25	-	1.0
4	-	<1.0
8	-	<1.0
16	-	1.0
24	-	2.25
48	-	5.20
72	+	4.93
96	+	5.67

+ = observed - = not observed

TABLE 51

VIRAEMIA IN CHICKENS INFECTED INTRAMUSCULARLY WITH $10^{7.5}$ $TCID_{50}$ OF THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Period post-infection (hours)	Age of chickens (days)		
	1	2-3	7
1	0/2	7/10	4/5
2	1/2	4/10	2/4
4	2/4	2/8	0/3
6	3/5	4/12	0/6
12	2/2	3/5	
18	2/2	1/3	
24	2/2	1/7	
30	1/2	1/5	
48	0/1	1/3	
66	0/1		
72	0/1	1/4	
96		0/2	

Numerator = number positive

Denominator = number of samples tested

TABLE 52

MEAN VIRUS TITRES AND STANDARD ERRORS IN THE MUSCLE TISSUE AT THE SITES OF INOCULATION, SPINAL CORDS AND BRAINS OF CHICKENS INFECTED INTRAMUSCULARLY WITH THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Tissue	Age of chickens (hours)		
	10	22	37
Muscle	2.3 ± 0.15	2.1 ± 0.10	1.7 ± 0.00
Spinal cord	< 1.00	2.2 ± 0.00	1.9 ± 0.34
Brain	1.97 ± 0.15	2.1 ± 0.23	1.7 ± 0.05

TABLE 53

DEVELOPMENT OF VIRUS TITRES IN THE TISSUES OF CHICKENS LESS THAN 24 HOURS OF AGE INFECTED INTRAMUSCULARLY WITH $10^{7.5}$ $TCID_{50}$ OF THE McFERRAN STRAIN OF AUJESZKY'S VIRUS

Period of post-infection (hours)	Muscle	Blood	Spinal Cord	Brain
0	5.5			
0.50	2.5	<1.0	<1.0	<1.0
0.75	2.0			
1	2.8	<1.0	<1.0	<1.0
2	3.0	2.0	<1.0	<1.0
3		1.3		
4	5.0	1.3	<1.0	<1.0
6	4.5	1.3		
8	4.5	1.3	<1.0	<1.0
12	4.5	1.3	<1.0	<1.0
18		2.2	1.3	<1.0
24	4.5	2.2	3.2	<1.0
48	4.5	<1.0	3.8	2.0
72		<1.0	3.8	2.0
96		<1.0		2.0
120	5.0	<1.0	2.5	4.5

TABLE 54

VIRUS TITRES IN THE TISSUES OF CHICKS INFECTED WITH THE HUNGARIAN STRAIN OF AUJESZKY'S VIRUS

Tissue	Intramuscular		Intracerebral
	$10^{7.75}$ $TCID_{50}$	$10^{3.75}$ $TCID_{50}$	$10^{7.15}$ $TCID_{50}$
Brain	5.30	3.90	4.67
Spinal Cord	5.00	5.00	3.00
Heart	3.00	1.80	3.00
Lung	3.00	2.00	3.00
Liver	3.00	2.00	2.50
Spleen	3.00	2.00	2.50
Kidney	3.00	1.80	2.50
Muscle at sites of inoculation	7.00	4.30	ND

TABLE 55

VIRAEMIA IN CHICKS INFECTED WITH THE HUNGARIAN STRAIN OF AUJESZKY'S VIRUS*

Hours post-infection	Day-old		Nine days old	
	Intramuscular	Intracerebral	Intramuscular	Intracerebral
0.5	-	-	-	+
1	-	+		
3	-	+	±	-
4	-	ND	ND	ND
6	+	+	+	+
9	-	+	ND	ND
24	+	+	-	-
48	+	+	-	-
72	-	-	-	-

* The virus had an infectivity titre of $10^{8.45}$ $TCID_{50}$/ml for PK15 cells

+ = virus recovered - = virus not recovered

± = virus recovered from some samples

ND = not done

TABLE 56

TITRES* OF SPECIFIC ANTIGENS IN THE TISSUES OF YOUNG CHICKS INFECTED WITH THE HUNGARIAN STRAIN OF AUJESZKY'S VIRUS

Tissue	Route of infection		
	Intramuscular		Intracerebral
	$10^{2.8}$ $TCID_{50}$	$10^{6.8}$ $TCID_{50}$	$10^{6.2}$ $TCID_{50}$
Spinal cord	3.2	2.9	3.2
Brain	2.3	2.0	2.9
Heart	2.0	2.0	2.3
Lung	2.0	2.0	2.3
Liver	<2.0	<2.0	<2.0
Spleen	<2.0	<2.0	<2.0
Kidney	2.3	<2.0	2.3
Muscle at the site of inoculation	2.0	2.0	

*Expressed as the negative logarithm of the number of CF units per ml.

TABLE 57

RESISTANCE TO INTRACEREBRAL CHALLENGE* OF CHICKENS PREVIOUSLY INOCULATED INTRAMUSCULARLY AT DIFFERENT AGES WITH THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Dose of virus in the primary inoculum ($log_{10}TC1D_{50}$/0.1 ml.)	Age (days) at primary and challenge infections											
	0.30 P	14 C	0.40 P	14 C	0.50 P	18 C	1.50 P	21 C	2.40 P	21 C	8 P	28 C
6.8	20/20	-	6/7	0/1	21/30	0/6	4/15	0/9	0/3	0/2	0/4	0/3
5.8	12/14	0/2	7/8	0/1	5/21	0/15	0/7	0/7	0/3	0/3	0/4	0/3
4.8	9/14	0/5	6/8	0/2	4/21	1/14	3/7	0/4	0/3	0/3	0/4	1/4
3.8	4/8	0/4	1/8	0/6	1/15	1/10	1/7	0/6	0/3	0/3	0/4	3/4
2.8	0/8	0/7	0/6	0/4	0/13	0/12	0/7	0/7	0/3	0/3	0/4	0/3
1.8	0/7	0/5	0/6	0/5	0/10	3/8	0/5	1/4	0/3	1/3	0/4	2/4
0.8	0/3	1/2	0/3	0/3	0/10	3/9	0/5	2/5			0/4	4/4
0.08	0/3	1/2			0/10	6/10	0/2	2/2			0/4	2/4
0.008	0/3	2/3			0/10	5/10	6/2	1/1			0/4	3/4
0.0008	0/3	1/3			0/10	5/9	0/2	1/2			0/4	2/3
Challenge controls	-	20/28	-	5/5	-	30/40	-	9/10	-	3/3	-	4/5

Numerator = number dead Denominator = number inoculated

P = Primary inoculation C = Challenge infection

*The $TC1D_{50}$ of virus used as undiluted in intracerebral challenge was $10^{7.5}$ per ml.

TABLE 58

REGRESSIONS OF THE PERCENTAGE OF RECOVERIES FROM INTRACEREBRAL CHALLENGE INFECTION ON THE DOSE OF VIRUS IN THE PRIMARY INOCULUM IN CHICKENS PREVIOUSLY INFECTED INTRAMUSCULARLY

Age (days) at primary infection	Regression	d.f.	Correlation coefficient
0.30	$\hat{Y} = 56.06 + 12.63.X$	7	0.878**
0.40	$\hat{Y} = 43.46 + 13.10.X$	7	0.696*
0.50	$\hat{Y} = 53.46 + 9.31.X$	8	0.923**
1.50	$\hat{Y} = 27.93 + 14.49.X$	8	0.864**
2.40	$\hat{Y} = 78.00 + 4.71.X$	4	0.654
8.00	$\hat{Y} = 32.12 + 11.75.X$	8	0.734*

TABLE 59

RESISTANCE TO INTRACEREBRAL CHALLENGE* OF CHICKENS PREVIOUSLY INOCULATED SUBCUTANEOUSLY AT DIFFERENT AGES WITH THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Dose of virus in the primary inoculum ($log_{10}TC1D_{50}$/0.2 ml.)	Age (hours) at subcutaneous infection							
	13		21		34		60	
	P	C	P	C	P	C	P	C
6.3	14/17	0/2	4/7	0/3	3/8	0/1	1/8	0/4
5.3	10/12	0/2	1/2	0/1	0/3	0/3	0/5	0/5
4.3	8/12	0/4	0/2	0/2	0/3	0/3	1/5	0/4
3.3	4/12	0/5	0/2	0/2	0/3	0/3	0/5	0/5
2.3	2/10	2/8	0/2	0/2	0/3	1/3	0/5	0/5
1.3	3/11	1/9	0/2	0/2	0/3	0/3	0/5	0/5
0.3	0/12	3/10	0/2	0/2	0/3	0/3	0/5	4/5
0.03	0/12	6/9	0/2	1/2	0/3	0/3	0/5	2/4
0.003	0/12	6/10	0/2	2/2	0/3	2/3	0/5	2/4
Challenge controls	-	14/16	-	14/16	-	14/16	-	14/16

Numerator = number dead Denominator = number inoculated

P = Primary infection C = Challenge infection

*The virus was used undiluted and had an infectivity titre of $10^{7.0}TC1D_{50}$ per ml. for PK15 cells.

TABLE 60

RESISTANCE TO INTRACEREBRAL CHALLENGE* OF CHICKENS PREVIOUSLY INOCULATED INTRACEREBRALLY AT DIFFERENT AGES WITH THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Dose of virus in the primary inoculum (log_{10} $TC1D_{50}$/0.05 ml.)	Age (days) at first and second intracerebral infection											
	1	33	7	28	14	39	21	53	28	46	46	74
	P	C	P	C	P	C	P	C	P	C	P	C
6.9	10/10	-	5/5	-	7/8	0/1	6/7	0/1	9/13	0/3	6/10	0/4
5.9	10/10	-	3/5	0/2	5/8	0/3	7/8	0/1	12/13	0/1	4/7	0/2
4.9	9/10	0/1	4/5	0/1	7/8	0/1	5/10	0/4	10/15	0/4	4/8	0/2
3.9	9/10	0/1	3/5	0/2	3/8	0/4	3/5	0/1	7/10	0/3	1/8	0/4
2.9	8/10	0/2	3/5	0/2	2/8	2/3	1/5	0/3	3/10	1/5	1/8	1/5
1.9	4/10	0/5	3/5	0/2	2/8	2/5	0/5	4/4	2/10	2/5	1/7	2/6
0.9	7/10	2/3	0/5	3/5	0/8	7/8	-	-	0/5	1/4	0/5	1/4
0.09	0/10	6/9	-	-	0/8	5/6	-	-	0/5	2/4	0/5	3/4
0.009	-	-	-	-	0/5	2/4	-	-	0/5	4/5	0/5	1/2
0.0009	-	-	-	-	0/4	2/4	-	-	0/5	3/5	0/5	3/5
Challenge controls	-	7/7	-	12/15	-	17/23	-	4/4	-	20/30	-	15/23

Numerator = number dead Denominator = number inoculated

P = Primary infection C = Challenge infection

*The $TC1D_{50}$ of virus used as undiluted in the challenge infection was $10^{7.5}$ per ml.

TABLE 61

REGRESSIONS OF THE PERCENTAGES OF RECOVERIES FROM INTRACEREBRAL CHALLENGE INFECTION ON THE DOSE OF VIRUS IN PRIMARY INOCULUM IN CHICKENS PREVIOUSLY INFECTED SUBCUTANEOUSLY

Age (hours) at primary infection	Regression	d.f.	Correlation coefficient
13	$\hat{Y} = 60.53 + 9.65.X$	7	0.822*
21	$\hat{Y} = 61.28 + 8.58.X$	7	0.589
34	$\hat{Y} = 78.48 + 4.05.X$	7	0.415
60	$\hat{Y} = 54.94 + 0.75.X$	7	0.739*

TABLE 63

DEVELOPMENT OF RESISTANCE TO INTRACEREBRAL CHALLENGE IN CHICKENS PREVIOUSLY EXPOSED TO AUJESZKY'S VIRUS INTRAMUSCULARLY

Day of Challenge	Age (days) at first inoculation							
	2				14			
	Infected		Controls		Infected		Controls	
	Responses	Percentage of survivors	Responses	Percentage of survivors	Responses	Percentage of survivors	Responses	Percentage of survivors
1	1/22	5	16/16	0	8/20	40	6/20	30
4	21/23	91	19/19	0	19/20	95	7/20	35
8	17/18	94	4/17	23	18/21	86	9/20	45
11	15/16	94	7/24	29	31/33	94	16/25	64
14	3/3	100	10/13	71	13/13	100	7/19	37

Numerator = number survived

Denominator = number tested

TABLE 64

NEUTRALISING ANTIBODY TITRES IN THE SERA OF CHICKENS INFECTED WITH THE McFERRAN STRAIN OF AUJESZKY'S VIRUS

Number of inoculations	Period of sampling (Weeks P.I)	Number tested	Mean titre (log_{10} units per ml.) + S.E.
2	3 - 5	6	1.95 ± 0.39
	6 - 9	5	3.02 ± 0.61
	10 - 12	3	2.62 ± 0.13
3	7 - 11	7	2.40 ± 0.02
	12 - 16	9	1.97 ± 0.37
4	14 - 22	7	2.29 ± 0.35

TABLE 65

COMPARISON OF NEUTRALISING ANTIBODY RESPONSES OF CHICKENS INOCULATED WITH INACTIVATED OR LIVE McFERRAN STRAIN OF AUJESZKY'S VIRUS : SIGHTING EXPERIMENT

Primary inoculation					Secondary inoculation				
Route	Inoculum	Number of doses	Number of chickens	Antibody titre*	Route	Inoculum	Number of doses	Number of chickens	Antibody titre*
Intravenous	Inactivated virus	2	3	<1.6	Intravenous	Live virus	1	3	2.0
Subcutaneous	"	2	3	<1.6	"	"	1	3	2.5
Intravenous	Live virus	2	3	<1.6	"	Inactivated virus	1	3	2.4
Subcutaneous	"	2	3	1.6	"	"	1	3	1.6

*Expressed as the negative logarithm of the number of neutralising antibody units per ml.

TABLE 66

COMPARISON OF NEUTRALISING ANTIBODY RESPONSES OF CHICKENS INOCULATED WITH ONE DOSE OF LIVE OR INACTIVATED VIRUS AND THEN CHALLENGED INTRACEREBRALLY : CONFIRMATORY EXPERIMENT

Virus strain	Primary inoculum	Titre after intracerebral challenge
McFerran	Live virus	3.0
		3.1
		2.5
		2.1
	Inactivated virus	1.9
		1.7
Hungarian	Live virus	2.2
		3.2
		2.2
	Inactivated virus	1.9

TABLE 67

COMPARISON OF NEUTRALISING ANTIBODY RESPONSES OF CHICKENS FIRST INOCULATED INTRAMUSCULARLY WITH TWO DOSES OF LIVE OR INACTIVATED AUJESZKY'S VIRUS AND THEN CHALLENGED INTRACEREBRALLY: CONFIRMATORY EXPERIMENT

Virus strain	Inoculum	Antibody titres (log_{10} units per ml.)		
		Intramuscular inoculation		Intracerebral challenge
		After first dose	After second dose	
McFerran	Live virus	1.30	1.60	3.20
		1.30	2.40	3.20
		1.50	2.50	3.20
	Inactivated virus	<1.00	<1.00	1.60
		<1.00	<1.00	1.50
Hungarian	Live virus	1.30	1.80	2.50
		1.30	1.30	3.40
	Inactivated virus	<1.00	<1.00	1.70
		<1.00	<1.00	2.70

TABLE 68

COMPARISON OF NEUTRALISING ANTIBODY TITRES FOLLOWING INTRACEREBRAL CHALLENGE IN CHICKENS PREVIOUSLY INOCULATED WITH LIVE OR INACTIVATED McFERRAN OR HUNGARIAN STRAINS OF AUJESZKY'S VIRUS

Inoculum	Differences in titres (log_{10} units per ml)	S.E. of the difference	d.f.	t	P
Live and inactivated McFerran Strain	1.21	0.226	9	5.354**	<0.01
Live and inactivated Hungarian Strain	0.77	0.354	6	2.175*	<0.05
Live McFerran and live Hungarian Strains	0.19	0.902	10	0.211	>0.50
Inactivated McFerran and inactivated Hungarian Strains	0.25	0.179	5	1.395	>0.20

TABLE 69

DEVELOPMENT OF NEUTRALISING ANTIBODY TITRES IN CHICKENS INOCULATED WITH THREE DOSES OF LIVE McFERRAN STRAIN OF AUJESZKY'S VIRUS

Chicken	Weeks post-infection						
	*1	2*	3	4	5	6*	8
1	<1.0	<1.0	2.1	2.5	1.9	2.5	3.7
2	<1.0	<1.0	1.9	1.9	1.5	1.6	3.7
3	<1.0	<1.0	1.9	<1.0	1.3	1.0	3.1
4	<1.0	<1.0	1.3	1.3	1.0	1.3	2.8
5	<1.0	<1.0		1.3		<1.0	
Median	<1.0	<1.0	1.9	1.3	1.45	1.3	3.7

*Inoculations were given on day 0 and 2 and 6 weeks.

TABLE 70

PERSISTENCE OF NEUTRALISING ANTIBODIES IN CHICKENS GIVEN MULTIPLE DOSES OF LIVE McFERRAN STRAIN OF AUJESZKY'S VIRUS

Chicken	Days post-infection			
	560*	567	574	581
1	1.9	3.1	3.7	3.1
2	1.9	3.1	3.1	3.7
3	1.9	3.7	3.7	3.7
4	1.9	1.9	3.4	
Median	1.9	3.1	3.55	3.7

*Infected intravenously with live virus

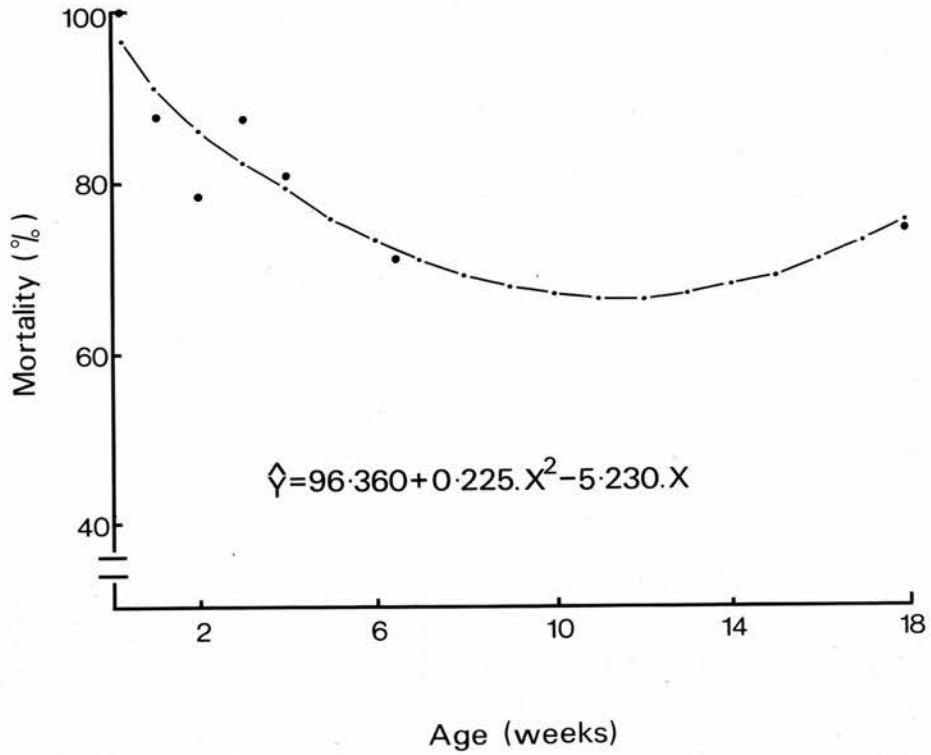
TABLE 71

EFFECT OF ADDITION OF GUINEA PIG COMPLEMENT TO VIRUS-SERUM MIXTURES ON THE NEUTRALISING ANTIBODY TITRES TO AUJESZKY'S VIRUS IN CONVALESCENT CHICKEN SERA

Without complement	With complement
< 1.0	< 1.0
< 1.0	< 1.0
< 1.0	< 1.0
< 1.6	< 1.6
1.3	1.3
2.2	2.2
2.5	2.5
< 1.0	1.3
< 1.6	1.6
< 1.0	1.7
1.4	1.9
3.1	3.4
3.1	2.7
2.9	2.6
3.0	2.5
Number positive 8	11
Median of positives 2.7	2.20

Figs. 56 and 57. - Symptoms of dyspnoea and paralysis in young chicks infected intramuscularly with the McFerran strain of Aujeszky's virus.

Figs. 58 and 59. - The relationships between the age at the time of infection and the percentage of mortality and the mean time of death in chickens intracerebrally infected with a high dose of the McFerran strain of Aujeszky's virus.


100
80
60
40
Mortality (%)
$\hat{Y}=96{\cdot}360+0{\cdot}225.X^2-5{\cdot}230.X$
2
6
10
14
18
Age (weeks)

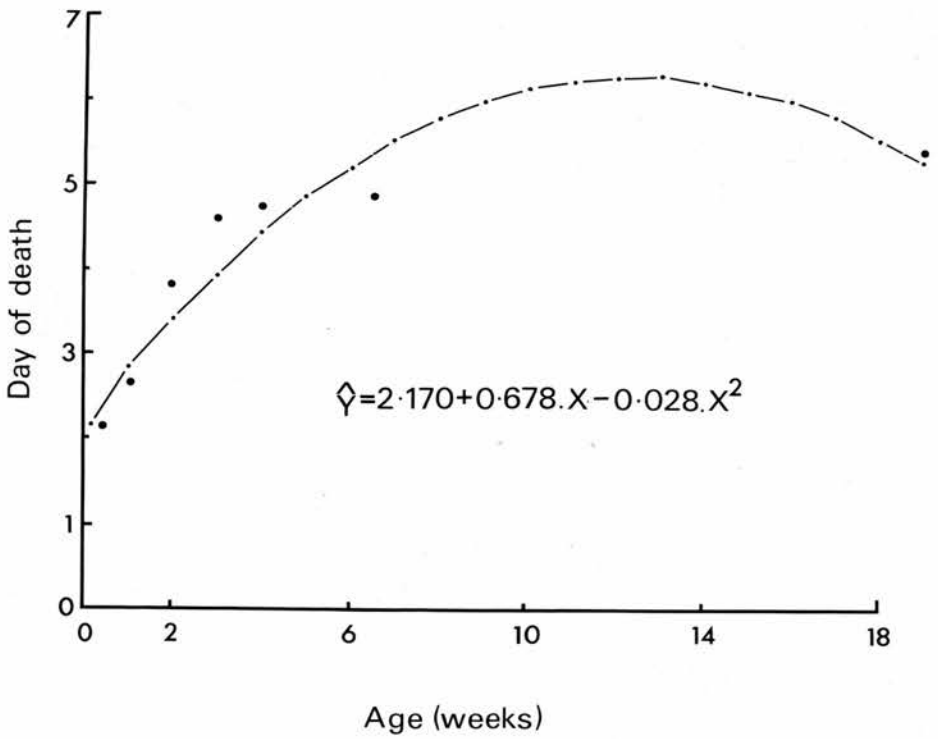
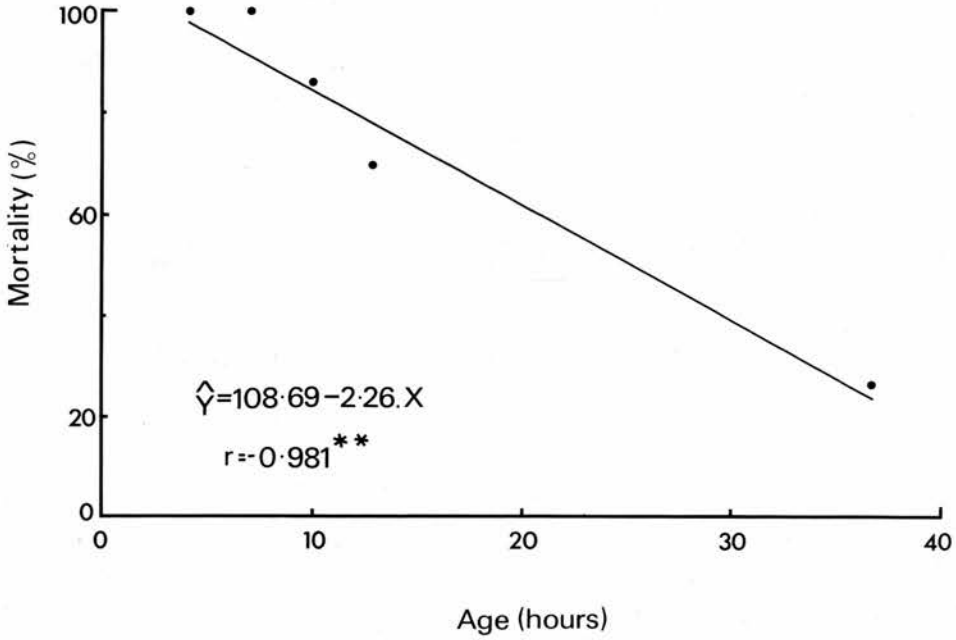

7
5
3
1
0
Day of death
$\hat{Y}=2{\cdot}170+0{\cdot}678.X-0{\cdot}028.X^2$
0
2
6
10
14
18
Age (weeks)

Fig. 60. - The relationship between age and the percentage of deaths in young chicks infected intramuscularly with the McFerran strain of Aujeszky's virus.

Fig. 61. - A comparison of the influence of age on the percentage of mortality in young chicks infected intramuscularly or subcutaneously with the McFerran strain of Aujeszky's virus.


100
60
20
0
Mortality (%)
$\hat{Y}$=108·69−2·26.X
r=-0·981**
0
10
20
30
40
Age (hours)

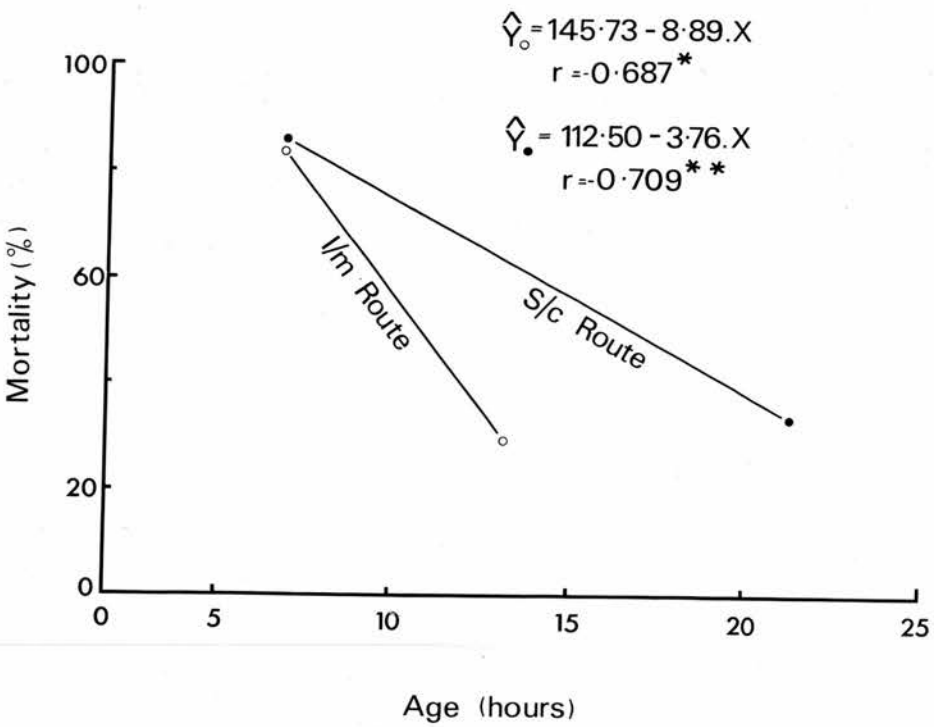

$\hat{Y}_{\circ}$=145·73 - 8·89.X
r=-0·687*
$\hat{Y}_{\bullet}$= 112·50 - 3·76.X
r=-0·709**
100
60
20
0
Mortality (%)
I/m Route
S/c Route
0
5
10
15
20
25
Age (hours)

Fig. 62. - The relationship between the dose of virus and the percentage of mortality in chickens of different ages infected intracerebrally with the McFerran strain of Aujeszky's virus.

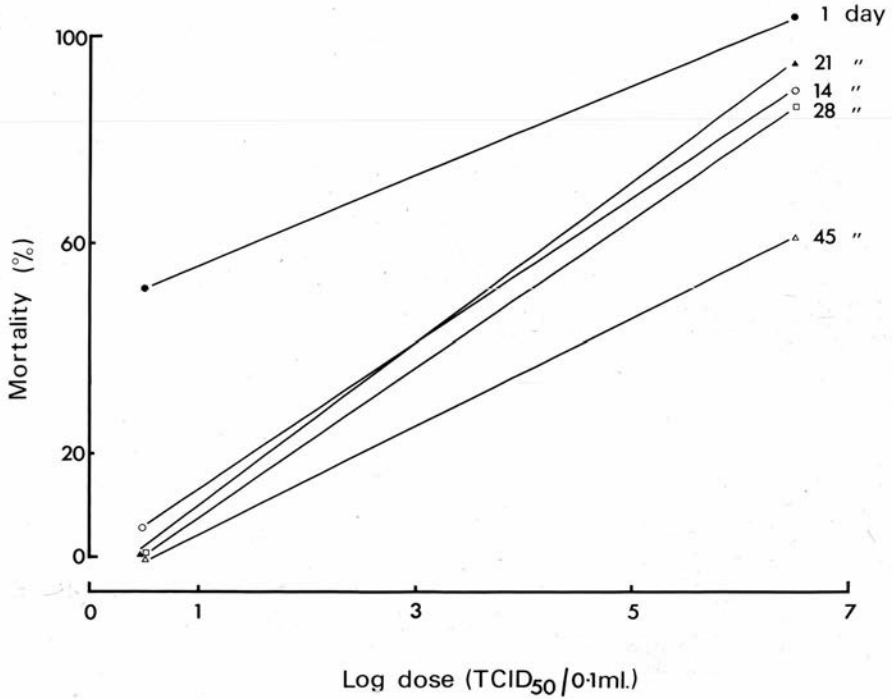
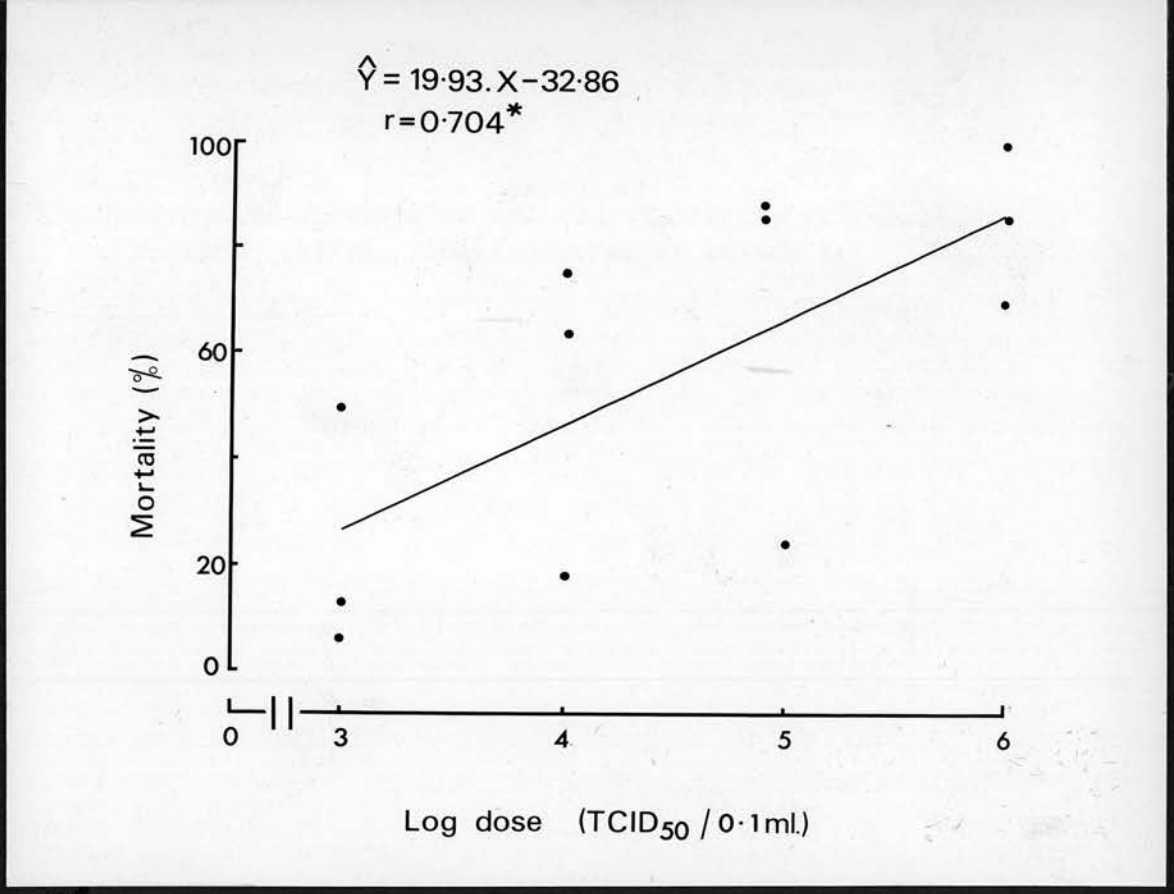

100
60
20
0
Mortality (%)
0
1
3
5
7
Log dose (TCID50/0·1ml.)
1 day
21 "
14 "
28 "
45 "

Fig. 63. - The effect of the dose of virus on the percentage of deaths in chicks infected intramuscularly with the McFerran strain of Aujeszky's virus.

Fig. 64. - The effect of the dose of virus on the percentage of deaths in subcutaneously infected chicks.


$\hat{Y}$ = 19·93. X − 32·86
r = 0·704*
100
60
20
0
Mortality (%)
0
3
4
5
6
Log dose (TCID50 / 0·1ml.)

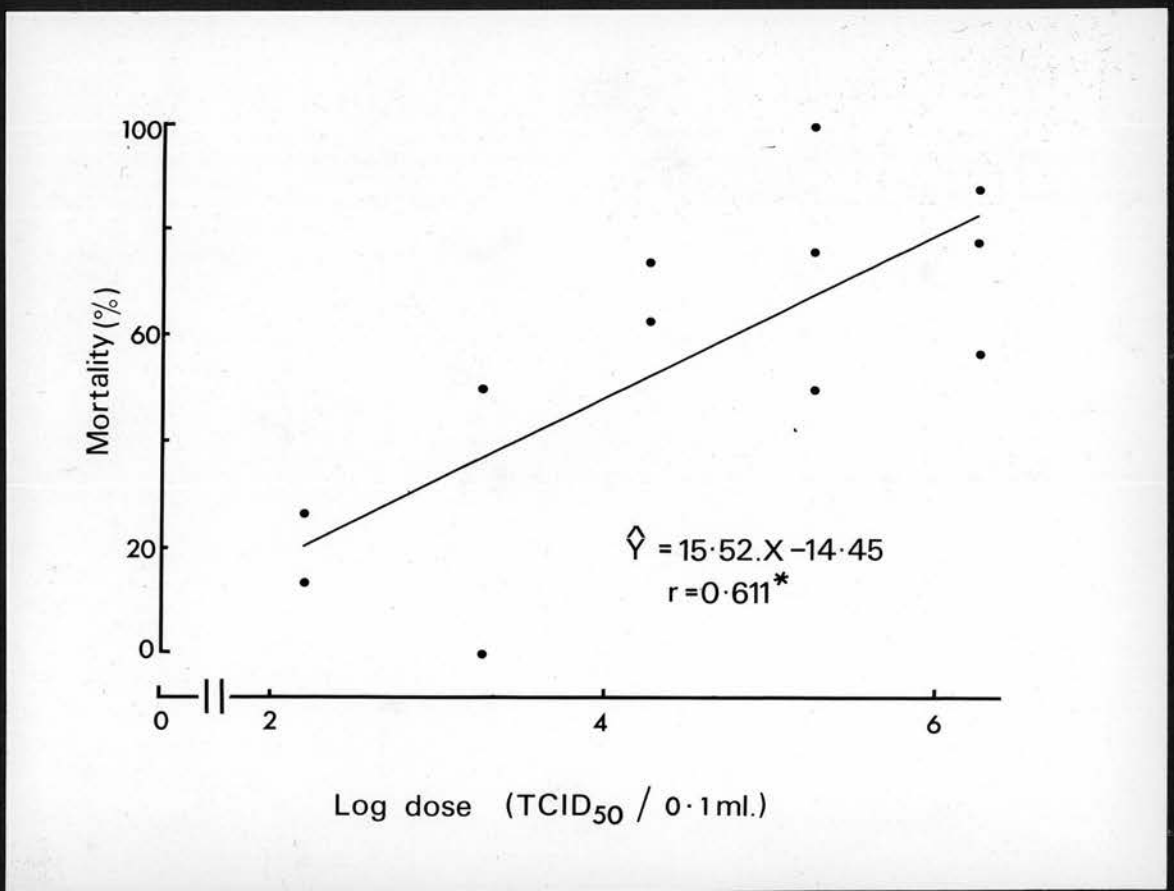

100
60
20
0
Mortality (%)
$\hat{Y}$ = 15·52. X − 14·45
r = 0·611*
0
2
4
6
Log dose (TCID50 / 0·1ml.)

Fig. 65. - The influence of age on the percentage of mortality in young chicks intracerebrally infected with $10^{6.9}$ $TCID_{50}$ of the Weybridge strain of Aujeszky's virus.

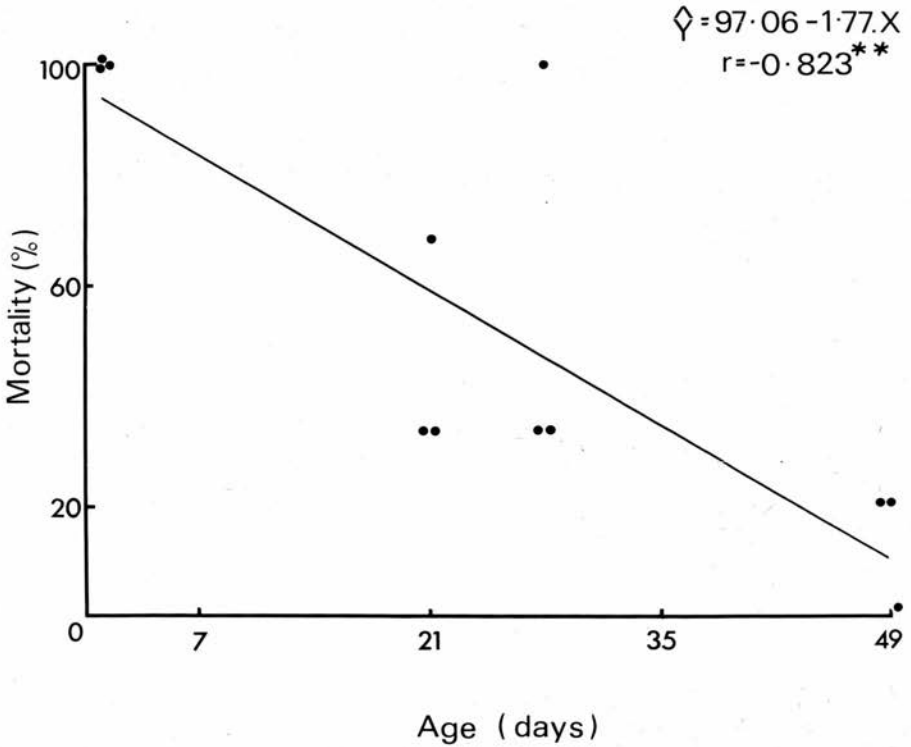
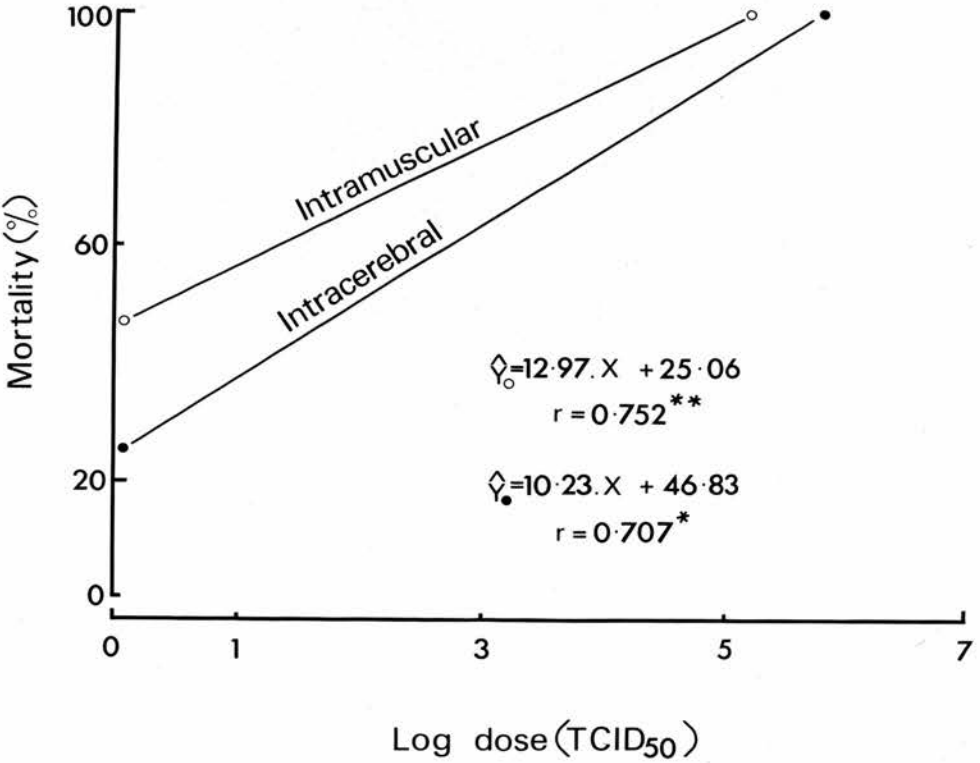

$\hat{Y} = 97 \cdot 06 - 1 \cdot 77.X$
$r = -0 \cdot 823^{**}$
100
60
20
0
Mortality (%)
7
21
35
49
Age (days)

Fig. 66. - The effect of the dose of virus on the percentage of deaths in day-old chicks infected intramuscularly or intracerebrally with the Hungarian strain of Aujeszky's virus.

Fig. 67. - The relationship between the dose of virus and the mean time of death in young chicks infected intracerebrally or intramuscularly with the Hungarian strain of Aujeszky's virus.


100
60
20
0
Mortality (%)
Intramuscular
Intracerebral
$\hat{Y}_o$=12·97. X + 25·06
r = 0·752**
$\hat{Y}_\bullet$=10·23. X + 46·83
r = 0·707*
0
1
3
5
7
Log dose (TCID50)

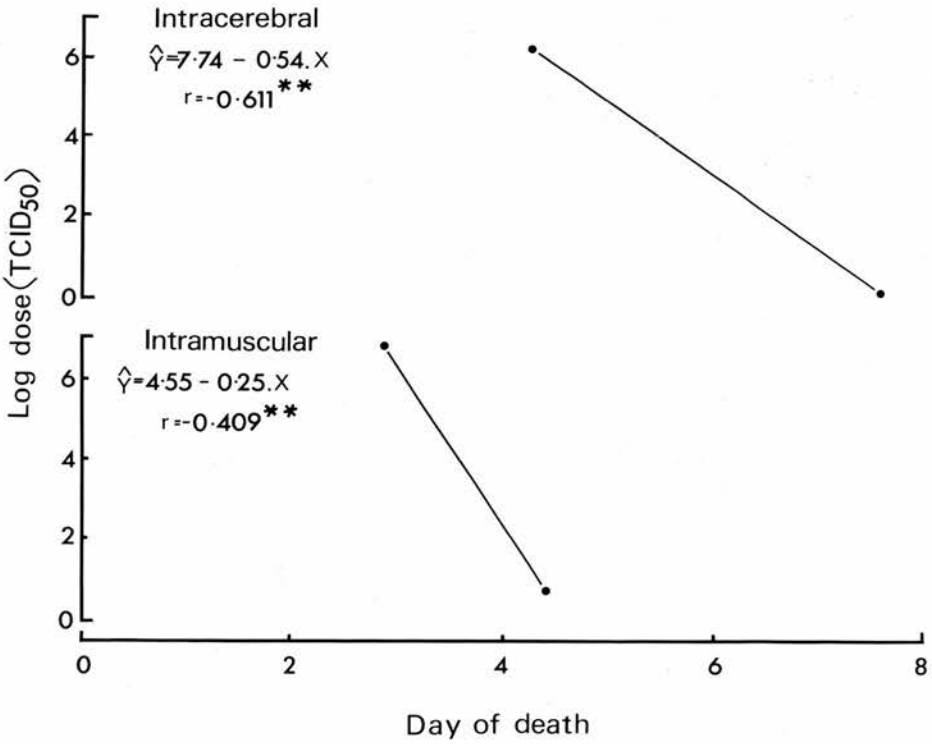

Intracerebral
$\hat{Y}$=7·74 - 0·54. X
r=-0·611**
Intramuscular
$\hat{Y}$=4·55 - 0·25. X
r=-0·409**
Log dose (TCID50)
6
4
2
0
0
2
4
6
8
Day of death

Fig. 68. - The influence of the dose of virus on the percentage of deaths in one day-old and two days' old chicks infected intramuscularly with the Hungarian strain of Aujeszky's virus.

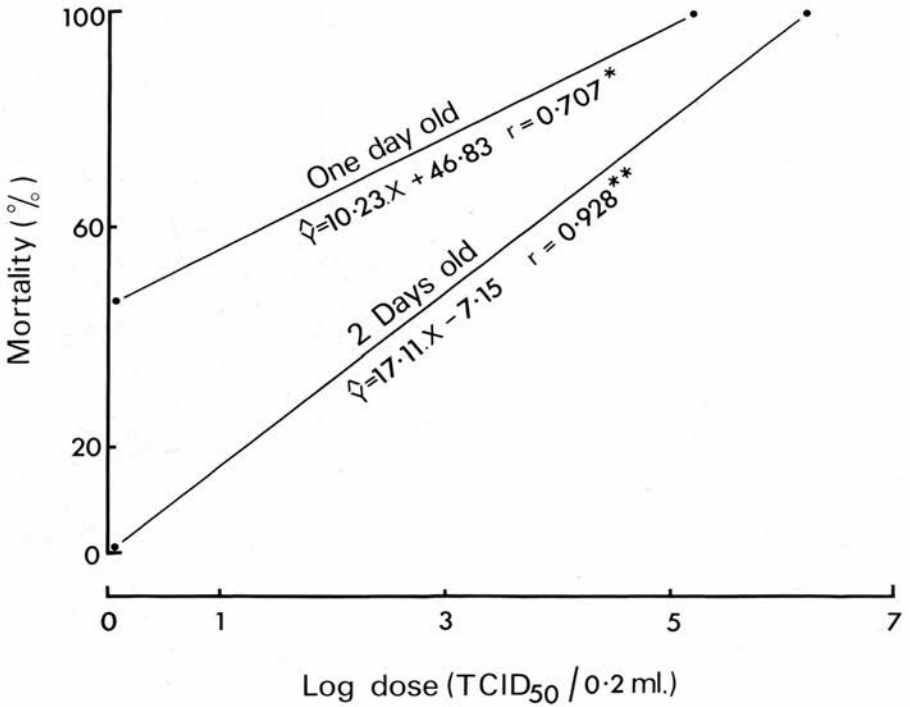
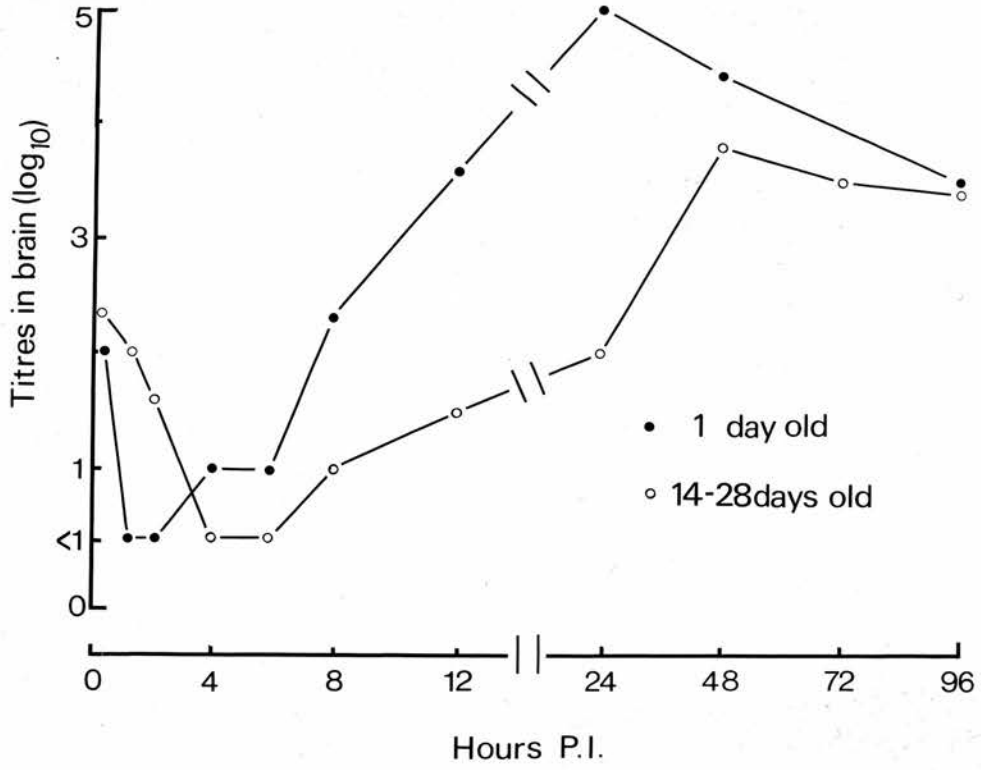
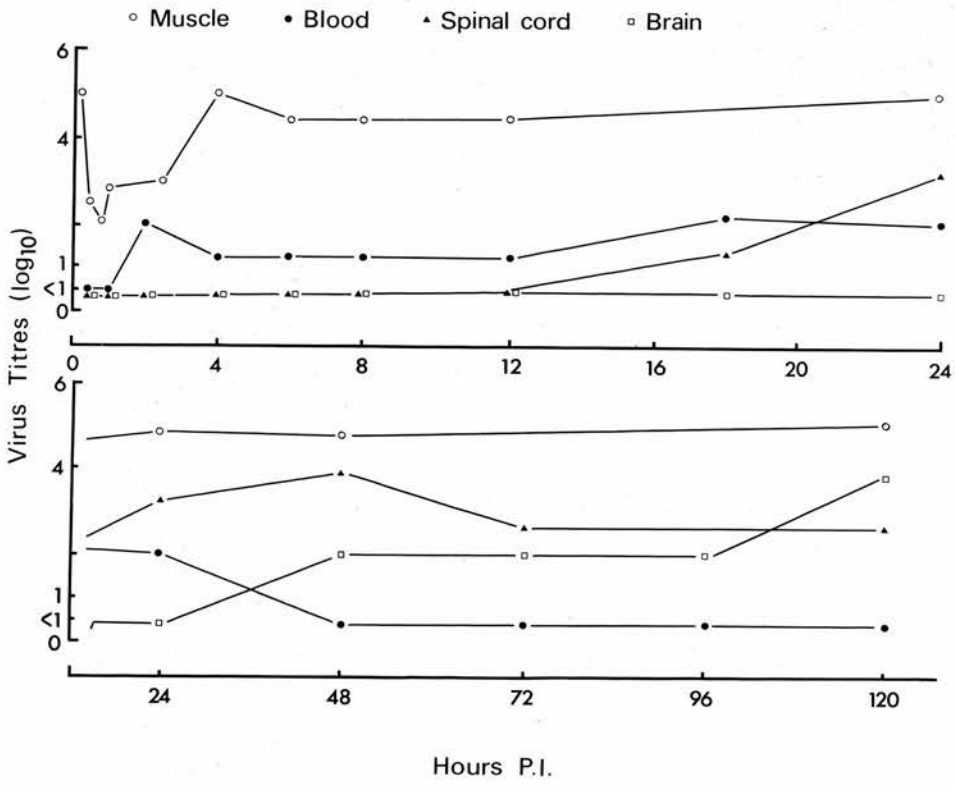

100
60
20
0
Mortality (%)
One day old
Ŷ=10·23X + 46·83 r = 0·707*
2 Days old
Ŷ=17·11X − 7·15 r = 0·928**
0
1
3
5
7
Log dose (TCID50 / 0·2 ml.)

Fig. 69. - Evolution of the virus titres in the brains of chicks of different ages infected intracerebrally with the McFerran strain of Aujeszky's virus.

Fig. 70. - Development of virus titres in the tissues of chicks less than 24 hours of age infected intramuscularly with the McFerran strain of Aujeszky's virus.


Titres in brain (log_{10})
5
3
1
<1
0
0
4
8
12
24
48
72
96
Hours P.I.
1 day old
14-28days old


Muscle
Blood
Spinal cord
Brain
Virus Titres (log_{10})
6
4
1
<1
0
0
4
8
12
16
20
24
24
48
72
96
120
Hours P.I.

PATHOGENICITY FOR MICE

CLINICAL SYNDROME

Effect of Route of Inoculation: Laboratory-bred mice were highly susceptible to experimental infection with Aujeszky's virus. Whereas all mice infected intracerebrally, intravenously, intramuscularly or intraperitoneally developed symptoms of the disease and died, some did not react clinically when the virus was instilled into the anterior nares or into the conjunctival sac (Table 72). Nervous signs such as muscular tremors, locomotor incoordination and regional or generalized pruritus of the skin were the guide lines used in assessing the approximate duration of the incubation period.

The mean incubation periods ranged from 28 to 83 hours (Table 72) and the differences were apparently related to the routes of infection. For instance, the mean incubation period in mice infected intravenously was lower than that in those infected intramuscularly or intraperitoneally, but the differences were not significant ($t = 1.084$; $P > 0.30$ and $t = 1.364$; $P > 0.20$ respectively). There were no differences in the time of appearance of clinical symptoms in mice inoculated by the latter routes. Most mice infected intracerebrally developed clinical signs sooner than mice that were

inoculated intravenously despite the fact that the dose of virus injected into the brain was one $\log_{10}$ unit less than that introduced into the blood. Though pruritus of the skin was not observed in intracerebrally infected mice the animals were very ill after 24 to 36 hours and relapsed into a state of coma before they died. On the other hand, those inoculated intravenously or intraperitoneally developed a mild, intermittent but generalized pruritus of the skin that was accompanied by frequent "face-washing" movements of the fore-legs. The pruritus as well as oedema of the face were pronounced after 40 hours post-infection and most mice lay recumbent and in an exhausted condition.

The mean incubation periods were somewhat longer in mice injected intranasally and intraconjunctivally than in mice inoculated by other parenteral routes but the differences between the first two groups were not significant ($t = 0.715$; $P > 0.50$). Mice that were successfully injected intranasally became very sick by the end of 48 hours. There was no evidence of skin irritation but the animals lay curled up and appeared to be blind in both eyes. A few showed violent spasmodic movements shortly before death. Periorbital oedema and intense hyperaesthesia of the skin were more commonly observed on the 3rd or 4th day of infection in animals that were infected by instilling the virus into the

conjunctival sac. In the later stages of the infection the affected animal scratched and tore savagely at the skin of the face with its legs.

Clinical symptoms incident to intramuscular infection with the virus were generally spectacular from the 2nd day when the animal started to gnaw at the injected site. Soon the pruritus became so intense and persistent that the affected animals chewed furiously and continuously at the wound until they collapsed and died.

Effect of Age: To study the influence of the age of the animal on the duration of fatal infection and the percentage of mortality groups of adult, week-old and day-old mice were infected intramuscularly with different doses of Aujeszky's virus in 0.1 ml. amounts. The distribution of mortalities revealed that the susceptibility of mice to the virus was inversely related to age (Table 73). The LD_{50} for adult mice was significantly lower ($P < 0.01$) than that for week-old ($t = 2.820^{*}$; $P < 0.02$) and day-old mice ($t = 3.679^{**}$, $P < 0.01$). Between the latter groups the difference in the LD_{50} value was not significant ($t = 0.704$; $P > 0.4$), although one week-old animals had a slightly higher survival rate than day-old mice. The period of fatal infection in adult mice was also significantly longer than that in

day-old ($t = 2.793^{**}$; $P < 0.01$) and week-old mice ($t = 3.202^{**}$; $P < 0.01$) but there was no difference in the mean death periods in the two unweaned groups ($t = 0.004$; $P > 1.00$).

Effect of Dose of Virus: The results of a preliminary study (Table 73) suggested that irrespective of the age of the mice, the duration of fatal infection was directly related to the dose of the virus injected intramuscularly. The relationship was linear and significant ($F = 37.24^{**}$, 35.92^{**} and 19.13^{**}, d.f. 1, 33; 1, 29 and 1, 12 respectively; Fig. 71) such that mice inoculated with higher doses of the virus died earlier than those inoculated with lower doses. Furthermore, comparison of the three regressions of time of death on dose revealed that while the slopes were similar ($F = 0.44$, d.f. 2, 74) the levels were different ($F = 3.93^{*}$, d.f. 2, 74). Percentage of mortalities in the three groups of mice were likewise, dose dependent, (Table 73). The findings were confirmed in other trials in which larger numbers of adult mice were infected intramuscularly with 0.1 ml. amounts of virus containing different infective doses (Table 74). It was observed that the period of fatal infection was significantly related to the dose of virus used to infect the animals ($F = 93.03^{**}$, d.f. 1, 102) and

the line of best fit was linear (Fig 71).

VIRUS DISTRIBUTION

Irrespective of the route by which the mice were infected, virus was present in the brains of all the animals dying from the disease (Table 75). Whereas in intracerebrally or intraconjunctivally infected mice, the virus was isolated only from the brain, in intravenously inoculated animals the virus was recovered from all of the tissues tested. Intraperitoneal inoculation also entailed dissemination of the virus in a large number of tissues. The kidney was regularly infected after administration of the virus by the intramuscular, intravenous and intraperitoneal routes but only occasionally when the virus was introduced into the anterior nares.

In a limited study, evidence was sought for the presence of the virus in the blood of adult mice killed in the terminal stages of Aujeszky's disease induced by intramuscular infection. Whereas substantial amounts of virus were present in the brain (10^{3} to 4.2 $TCID_{50}$/ per gram) and kidney (10^{2} to 3.7 $TCID_{50}$/ per gram), the virus was detected in traces in the blood of only 1 of 4 mice.

In another study, adult mice were infected intramuscularly with 10^{-3} and 10^{-5} dilutions of a virus pool

with an infectivity titre of $10^{8.8}TCID_{50}$ doses/per ml. Two groups of dying mice and a third group that had survived the infection for 8 days were killed and their brains and kidneys were titrated for virus. The results (Table 76) show that whereas no virus was recovered from the survivors, the brains and kidneys of the dying mice contained different amounts of the virus.

LATERAL SPREAD OF INFECTION

In the course of the investigations on the influence of age of mice and size of inoculum on the mortality patterns, it was noted that some dams that had fed on the experimentally infected litters developed symptoms of Aujeszky's disease and died (Table 77). The virus was recovered from the brains and kidneys of the litters as well as from the brains of all mothers that had eaten the infected carcases of their offspring. The virus was also present in the lungs and hearts of 5 and 4 dams respectively and in the spleen in one case only. The virus was not isolated from the liver and kidney. Mothers of litters that survived the infection and also mothers that had not eaten their litters were unaffected.

RESISTANCE TO INFECTION

Adult mice were injected intramuscularly with 0.1 ml. amounts of 10-fold dilutions of Aujeszky's disease virus

equivalent to 0.04, 0.4, 4.0 and 40 LD_{50} doses. The survivors from each of these inoculations (Table 78) were divided into 3 groups and reinfected 15 days later with 4, 40 and 400 LD_{50} doses of the virus. Simultaneously, four groups of control mice were also inoculated with 0.4, 4 and 40 and 400 LD_{50} doses respectively. In the sensitized group, mortalities of 100 and 88 per cent were obtained when the infective doses were 400 and 40 LD_{50} doses respectively (Table 79). However, 75 per cent of mice survived the challenge infection when they were infected with only 4 LD_{50} doses of the virus despite the fact that this dose killed 7 out of 8 control mice.

The surviving mice were reinfected 14 days later with 4 LD_{50} doses of the virus along with 6 controls. The results (Table 79) suggest that when the infective dose was low a high percentage of the sensitized mice survived the challenge infection given at repeated intervals. The conclusion was justified by the results of the third challenge, which showed that 25 per cent of the sensitized mice were capable of withstanding no fewer than 400 LD_{50} doses of the virus. Further proof that resistance to reinfection may be broken with a higher dose of virus stemmed from the results of the final challenge that was carried out 13 days later with undiluted virus. None survived the challenge injection.

VIRUS-SPECIFIC TISSUE ANTIGENS

One hundred and thirty-eight preparations of minced fragments or suspensions of brain, heart, lung, liver, spleen and kidney from mice dying of experimentally induced Aujeszky's disease were diffused in agar gel against a pig anti-Aujeszky's hyperimmune serum. Positive control cell culture antigens were included in each test and the plates were incubated at 4, 20-25 and/or 37°C. No precipitation lines developed between the infected tissues and the serum in an observation period of seven days.

In another study, 2 specimens of brain tissue from mice that succumbed to infection with Aujeszky's disease virus were sonicated and the suspensions were clarified by centrifugation at 5,000 r.p.m. for 20 minutes. The supernatant fluids were spun at 10,000 r.p.m. for 30 minutes and the sediments harvested. The supernatant fluids from the above were subjected to a further centrifugation at 40,000 r.p.m. for 90 minutes. The deposits from the three runs were diffused in agar gel against pig anti-Aujeszky's serum and the plates were incubated. It was noted that whereas the pellets from the first and second centrifugation did not produce precipitation lines, one of the two pellets from the third centrifugation gave a faint line of precipitation

with the serum. The precipitation line formed a reaction of identity with that induced by the positive control antigens against the serum.

TABLE 72

MEANS AND STANDARD ERRORS OF RESPONSES OF ADULT MICE INFECTED WITH THE McFERRAN STRAIN OF AUJESZKY'S VIRUS

Parameter	Dose ($10^{TCID}50$/0.1 ml.) and routes of inoculation					
	5.2 Intracerebral	6.2 intravenous	6.2 intramuscular	6.2 intraperitoneal	6.0 intranasal	6.0 intraocular
Incubation period (hours)	28 ± 4.00	36 ± 12.01	47 ± 3.74	48 ± 2.83	61 ± 12.67	83 ± 18.71
Time of death (hrs.)	40 ± 0.00	53 ± 3.53	59 ± 2.38	63 ± 3.00	66 ± 6.28	91 ± 14.36
Number died/number infected	8/8	8/8	10/10	13/13	14/16	5/8
Percentage of mortality	100	100	100	100	88	63
LD_{50}	$10^{-5.50} \pm 0.41$	$10^{-3.00} \pm 0.54$	$10^{-4.50} \pm 0.36$	$10^{-4.15} \pm 0.38$	ND	ND
Skin pruritus	-	+	+	+	-	+

+ = observed - = not observed ND = not done

TABLE 73

THE EFFECT OF DOSE OF AUJESZKY'S VIRUS ON THE MORTALITY PATTERN IN MICE OF DIFFERENT AGES INFECTED INTRAMUSCULARLY

Age	Inoculum ($10^{TCID_{50}}$ doses per 0.1 ml.)	Days and number of deaths						No. died / No. used	LD_{50} ± S.E.	Mean death time (hrs.) ± S.E.
		1	2	3	4	5	6			
Day-old	6.8	-	8	-	-	-	-	8/3		45 ± 0.00
	4.8	-	11	2	-	-	-	13/13		52 ± 2.50
	2.8	-	-	9	-	-	-	9/9	$10^{-7.80}$ ± 0.46	72 ± 0.00
	0.8	-	-	3	1	1	-	5/6		86 ± 8.76
	0.008	-	-	-	-	-	-	0/9		0
Week-old	6.8	-	6	-	-	-	-	6/6		47 ± 0.00
	4.8	-	10	-	-	-	-	10/10		54 ± 1.06
	2.8	-	3	8	-	-	-	11/11	$10^{-7.24}$ ± 0.59	65 ± 3.39
	0.8	-	1	1	2	-	-	4/7		80 ± 7.42
	0.008	-	-	-	-	-	-	0/10		0
Adult	6.8	-	6	-	-	-	-	6/6		57 ± 0.00
	4.8	-	3	2	-	-	-	5/6		85 ± 2.93
	2.8	-	-	3	-	-	-	3/6	-4.70 ± 0.77	99 ± 1.88
	0.8	-	-	-	-	-	-	0/6		0
	0.008	-	-	-	-	-	-	0/6		0

- = no deaths

TABLE 74

MORTALITY PATTERN IN ADULT MICE INFECTED INTRAMUSCULARLY WITH DIFFERENT DOSES OF THE MCFERRAN STRAIN OF AUJESXKY'S VIRUS

Inoculum ($10^{TC1D}50$/0.1 ml.)	Responses	Percentage of mortality	Days of death									
			1	2	3	4	5	6	7	8	9	10
6.8	6/6	100	-	6	-	-	-	-	-	-	-	-
5.8	18/18	100	-	-	8	10	-	-	-	-	-	-
4.8	37/38	97	-	-	7	30	-	-	-	-	-	-
3.8	32/39	82	-	-	6	15	9	1	1	-	-	-
2.8	11/37	30	-	-	-	4	2	2	3	-	-	-
1.8	0/24	0	-	-	-	-	-	-	-	-	-	-
0.8	0/6	0	-	-	-	-	-	-	-	-	-	-
$LD_{50} \pm S.E.$	$10^{-4.60} \pm 0.16$											

- = no deaths Numerator = number died Denominator = number inoculated

TABLE 75

ISOLATION OF AUJESZKY'S VIRUS FROM THE TISSUES OF ADULT MICE EXPERIMENTALLY INFECTED BY DIFFERENT ROUTES

Route	Brain	Heart	Lung	Kidney	Liver	Spleen
Intracerebral	+	-	-	-	-	-
Intraocular	+	-	-	-	-	-
Intramuscular	+	-	-	+	-	-
Intranasal	+	+	+	(±)	-	-
Intraperitoneal	+	+	+	+	-	-
Intravenous	+	+	+	+	+	+

\+ = Virus isolated

\- = Virus not isolated

(±) = Virus occasionally isolated

TABLE 76

VIRUS TITRES IN THE TISSUES OF ADULT MICE INTRAMUSCULARLY INFECTED WITH AUJESZKY'S VIRUS

Inoculum ($10^{TC1D_{50}}$ per 0.1 ml.)	Day of death or destruction	N	Titre ($10^{TC1D_{50}}$ / ml.)	
			kidney	brain
4.80	4	3	3.50	4.50
2.80	7	3	2.50	3.00
	8	3*	0	0

N = Number of pools tested

* = Mice that survived the infection

TABLE 77

TRANSMISSION OF AUJESZKY'S VIRUS FROM EXPERIMENTALLY INFECTED LITTERS TO DAMS

Age of litter	Inoculum (10^{TC1D}_{50}/0.1 ml.)	Dam	Number in litter	Days and numbers of deaths									Number of unweaned mice eaten by dams
				1	2	3	4	5	6	7	8	9	
Day-old	6.8	A	8	-	8	A*	-	-	-	-	-	-	1
	4.8	B	13	-	11	2	-	B*	-	-	-	-	2
	2.8	C	9	-	-	9	-	-	-	C*	-	-	9
	0.8	D	6	-	-	3	1	1	-	-	-	D*	4
	0.008	E	9	-	-	-	-	-	-	-	-	-	0
Week-old	6.8	F	6	-	6	-	-	-	-	-	-	-	0
	4.8	G	10	-	10	-	-	-	-	-	-	-	0
	2.8	H	11	-	3	8	-	-	H*	-	-	-	6
	0.8	I	7	-	1	1	2	-	-	I*	-	-	1
	0.008	J	10	-	-	-	-	-	-	-	-	-	0

*Death of the dam - = no deaths

TABLE 78

RESPONSES OF ADULT MICE TO INTRAMUSCULAR INFECTION WITH AUJESZKY'S VIRUS

Dose of virus (10^{TCID}_{50}/0.1 ml.)	Responses	Number of survivor mice used for re-infection
4.8	25/25	0
3.8	19/25	6
2.8	6/25	17
1.8	0/25	24

Numerator = number dead Denominator = number used

TABLE 79

RESISTANCE OF ADULT MICE TO REINFECTION WITH THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS*

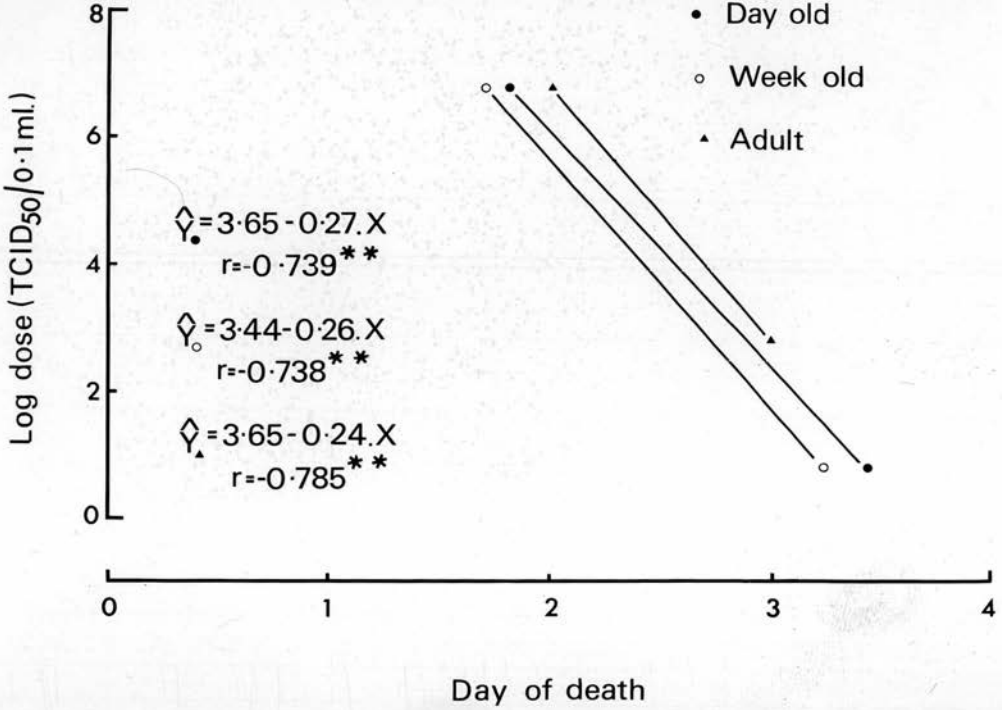
Dose (log TCID/0.1 ml.	Responses				Number of survivors			
	Infected mice			Control mice	Infected mice			Control mice
	(log $TCID_{50}$ doses)				(log $TCID_{50}$ doses)			
	3.8	2.8	1.8		3.8	2.8	1.8	
(i) First challenge:								
5.8	2/2	5/5	8/8	6/6	0	0	0	0
4.8	1/2	6/6	7/8	7/7	1	0	1	0
3.8	0/2	1/6	3/8	7/8	2	5	5	1
2.8				2/6				4
(ii) Second challenge:								
3.8(a)	1/3	0/5	1/6	6/6	2	5	5	0
3.8(b)		1/1	4/4			0	0	
(iii) Third challenge:								
5.8	2/2	3/5	4/5	5/5	0	2	1	0
(iv) Fourth challenge:								
7.8		2/2	1/1					

*The virus had an infectivity titre of $10^{7.8}$ $TCID_{50}$ doses per 0.1 ml. for PK15 cells.

Numerator = number dead. Denominator = number infected.

Second challenge: (a) = survivors from first challenge
(b) = surviving control mice.

Fig. 71. - The effect of dose of virus on the mean time of death in 3 groups of mice intramuscularly infected with the McFerran strain of Aujeszky's virus.


Day old
Week old
Adult
$\hat{Y}_{\bullet}$ = 3·65 - 0·27.X
r = -0·739**
$\hat{Y}_{\circ}$ = 3·44 - 0·26.X
r = -0·738**
$\hat{Y}_{\blacktriangle}$ = 3·65 - 0·24.X
r = -0·785**
Log dose (TCID50/0·1ml.)
8
6
4
2
0
0
1
2
3
4
Day of death

PATHOGENICITY FOR RATS

CLINICAL SYNDROME

Effect of Route of Inoculation: Symptoms of Aujeszky's disease in experimentally infected rats were not only very pronounced but were also of a more varied nature than in mice. Whereas excitability, lachrymation and facial oedema were constantly observed in rats infected by different routes, those infected intraocularly developed a marked conjunctivitis that was accompanied by intense pruritus of the skin around the eyes and diffuse oedema of the underlying tissues. Involvement of the conjunctiva was also a noteworthy feature in intramuscularly and intravenously infected rats (Fig. 72). In general, pruritus of the skin was less conspicuous in intracerebrally infected rats, although it was frequently observed in the animals infected by other routes. Subcutaneously or intramuscularly infected animals manifested an intense inflammatory reaction dominated by pruritus and oedema. The reaction was so intense that the fur in the affected regions became moist, discoloured and matted (Fig. 73). Diarrhoea was an additional feature of the disease in some rats, while male rats frequently showed orchitis and a viscid discharge from the preputial orifice (Fig. 74). There were no detectable abnormalities in the external genitalia of the females.

The mean periods of incubation and fatal disease ranged from 30 to 53 and 41 to 71 hours respectively (Table 80). Intracerebrally infected rats developed clinical symptoms and succumbed earlier than those inoculated by other peripheral routes. Differences in the mean incubation and mean death periods in the other groups apart from the intracerebrally infected rats, stemmed from the routes of infection of the virus ($F = 3.86^{**}$, d.f. 5, 75 and $F = 2.65^{*}$, d.f. 5, 75 respectively) and not the size of the inocula ($F = 2.86$, d.f. 1, 75 and $F = 1.14$, d.f. 1, 75; respectively). Intravenously infected rats developed clinical symptoms later than animals infected by other routes and the difference was significant ($F = 932^{**}$, d.f. 3, 38). Within the other groups, there were no differences in the mean incubation periods.

Differences in the mean duration of fatal infection were due to the fact that intranasally injected animals survived for a longer period than the other groups of rats; and these were significant ($F = 4.22^{**}$, d.f. 2, 37).

Effect of Dose: The influence of the size of the inoculum on the death pattern in rats was investigated by inoculating groups of adult rats intramuscularly with different doses of an aliquot of cell culture virus having an infective titre of $10^{8.8}$ $TCID_{50}$ per ml. The

results showed that the percentage of mortality and the time of death in rats were dose dependent (Table 81) and the relationship between the dose of virus injected and the length of period of fatal disease was linear and highly significant ($F = 32.10^{**}$; d.f. 1, 21; Fig. 75).

VIRUS DISTRIBUTION

Virus was present in the brains of all dying rats tested but the detection of virus in other tissues was related to the route of injection (Table 82). Whereas virus was isolated from the heart and lungs of rats injected by all except the intramuscular, subcutaneous and intraperitoneal routes, livers and spleens were infected only after intraperitoneal inoculations. In two groups of rats, intracerebral inoculation entailed infection of the adrenal gland. In intramuscularly infected rats, the muscle at the site of inoculation contained moderate amounts of virus.

The virus titres in brain tissues were significantly higher than those in the heart ($t = 2.868^{**}$, $P<0.05$) but were not significantly higher than the amount of virus either in the lung or kidney or in the adrenal gland (Table 83). In general, there were no differences in the virus titres in the different tissues other than the brain.

In a limited study, rats were injected intraperit-

oneally with $10^{7.3}$ $TCID_{50}$ doses of the McFerran strain of virus and housed in a metabolism cage. Samples of urine collected at different intervals post-infection until death were tested for virus by inoculation into PK15 cells; but no CPE occurred. Furthermore, 2 rabbits which were inoculated subcutaneously with 1 ml. each of pooled sample of urine collected from rats in the terminal stages of the disease showed no ill-effects and later proved susceptible to Aujeszky's disease when reinjected with cell culture virus.

VIRUS-SPECIFIC TISSUE ANTIGENS

Pooled 20 per cent suspensions of brain, lung and kidney of rats, which had succumbed to experimental Aujeszky's disease were inactivated at 56°C for 60 minutes and tested for CF antigens using positive and negative control rabbit anti-Aujeszky sera. Low titres of specific CF antigens were detected only in the brain suspensions (Table 84). Some suspensions revealed anticomplementary activities and also varying degrees of non-specific fixation of complement in the presence of negative control serum when 2-2.5 full units of complement were used. The non-specific reactions did not occur with higher units of complement and this facilitated the detection of specific CF antigens.

No immunodiffusion antigens were detected in the

pooled brain suspension that was concentrated approximately 8 times by dialysis against carbowax, although the titre of specific CF antigens in the preparation was 2.8 ml.

Eighty preparations of minced fragments or suspensions of brain, heart, lung, liver, spleen and kidney obtained from rats that died following various routes of infection were diffused in agar gel against a pig anti-Aujeszky's disease serum but no specific precipitation lines developed during an observation period of 7 days.

RESISTANCE TO REINFECTION

Adult rats that survived intramuscular infection with different doses of cell culture pseudorabies virus (Table 85) were reinfected a few days later with $10^{4.5}$ $TCID_{50}$ doses of the same pool of virus in 0.5 ml. amounts intramuscularly, along with 5 control rats. Most rats survived the challenge infection but the mortality in the control group was 40 per cent. The survivors from the test, including those of the control group were rechallenged a few days later with $10^{5.5}$ $TCID_{50}$ doses of the virus intramuscularly. All but one of the rats succumbed to the infection suggesting that the two previous exposures to the virus did not confer a degree of resistance sufficient to withstand high infective doses of the virus.

TABLE 80

MEANS AND STANDARD ERRORS OF RESPONSES OF ADULT RATS INFECTED WITH THE MCFERRAN STRAIN OF AUJESZKY'S VIRUS

Parameter	Dose (10^{TC1D}_{50} per 0.1 ml.) and routes of inoculation							
	5.2 Intracerebral	6.2 intravenous	6.2 intraperitoneal	6.2 intramuscular	6.2 subcutaneous	6.0 intranasal	6.0 intraocular	6.0 peroral
Incubation period (hrs.)	30.30±2.52	53.00±3.14	42.18±1.46	38.91±1.48	37.50±1.63	42.00±2.34	44.15±1.91	36.80±2.86
Time of death (hrs.)	40.80±0.53	70.00±5.28	66.47±1.29	60.10±1.48	63.38±1.72	71.53±2.85	64.65±1.13	64.20±2.54
Mortality/ number infected	10/10	12/12	11/11	11/11	8/8	15/15	20/21	5/5
Percentage of mortality	100	100	100	100	100	100	95	100
Skin pruritus	+	-	+	+	+	+	+	-

+ = observed ± = occasionally observed - = not observed

TABLE 81

INFLUENCE OF DOSE OF AUJESZKY'S VIRUS ON THE MORTALITY PATTERN IN ADULT RATS INFECTED INTRAMUSCULARLY

Inoculum ($_{10}^{TC}1D_{50}$ doses per 0.1 ml.)	Responses	Percentage of Mortality	Day of death					
			1	2	3	4	5	6
6.8	5/5	100	-	-	5	-	-	-
5.8	5/5	100	-	-	5	-	-	-
4.8	5/5	100	-	-	4	1	-	-
3.8	4/5	80	-	-	2	2	-	-
2.8	4/5	80	-	-	-	3	1	-
1.8	0/5	0	-	-	-	-	-	-
0.8	0/5	0	-	-	-	-	-	-
LD_{50}	$10^{-5.30} \pm 0.40$							

Numerator = number dead Denominator = number used

\- = no deaths

TABLE 82

DISTRIBUTION OF AUJESZKY'S VIRUS IN THE TISSUES OF ADULT RATS

EXPERIMENTALLY INFECTED BY DIFFERENT ROUTES

Route of inoculation	Brain	Heart	Lung	Kidney	Adrenal	Spleen	Liver	Muscle tissue at sites of inoculation
Intracerebral	+	+	+	-	+	-	-	ND
Intraocular	+	+	+	-	-	-	-	ND
Peroral	+	+	+	-	-	-	-	ND
Intranasal	+	+	+	-	-	-	-	ND
Intravenous	+	+	+	+	+	-	-	ND
Intraperitoneal	+	-	-	+	+	+	+	ND
Intramuscular	+	-	-	+	-	-	-	+
Subcutaneous	+	-	-	+	-	-	-	ND

+ = virus isolated - = virus not isolated ND = not done

TABLE 83

TITRES OF AUJESKY'S VIRUS IN THE TISSUES OF ADULT RATS EXPERIMENTALLY INFECTED BY DIFFERENT ROUTES.

Route of inoculation	Titres ($10^{TCID}50$ doses/per gram)							
	Brain	Heart	Lung	Kidney	Adrenal	Spleen	Liver	Muscle tissue at inoculation sites
Intracerebral	$4.0^{+}0.63$	$2.5^{+}0.36$	$2.5^{+}0.36$	0	$1.5^{+}0.31$	0	0	-
Intraocular	$4.0^{+}0.63$	$2.0^{+}0.63$	$2.0^{+}0.63$	0	0	0	0	-
Peroral	$2.8^{+}0.51$	$1.3^{+}0.48$	$4.8^{+}0.51$	0	0	0	0	-
Intranasal	$4.5^{+}0.36$	$2.0^{+}0.59$	$3.8^{+}0.51$	0	0	0	0	-
Intravenous	$3.0^{+}0.63$	$3.5^{+}0.44$	$3.5^{+}0.44$	$2.5^{+}0.44$	$3.5^{+}0.44$	0	0	-
Intraperitoneal	$1.5^{+}0.44$	0	0	$4.5^{+}0.44$	$2.5^{+}0.44$	$2.5^{+}0.44$	$2.5^{+}0.44$	-
Intramuscular	$1.0^{+}0.63$	0	0	$2.0^{+}0.39$	0	0	0	$3.5^{+}0.44$
Subcutaneous	$2.5^{+}0.44$	0	0	$1.5^{+}0.76$	0	0	0	0

- = not tested

0 = virus not isolated

TABLE 84

TITRES* OF SPECIFIC COMPLEMENT-FIXING ANTIGENS IN THE TISSUES OF ADULT RATS EXPERIMENTALLY INFECTED WITH AUJESZKY'S VIRUS

Serum	Units of C'	Brain (N=5)	Kidney (N=5)	Lung (N=3)
Positive control	2.0	2.4	2.0	2.3
	2.5	2.4	1.0	2.1
	3.0	2.2	1.0	1.0
	3.5	2.1	1.0	1.0
Negative control	2.0	2.0	1.9	1.8
	2.5	1.9	1.0	1.7
	3.0	1.0	1.0	1.0
	3.5	1.0	1.0	1.0
No serum	2.0	2.2	1.0	1.0
	3.0	1.0	1.0	1.0

*Mean values expressed as the logarithm of the CF units per ml.

N = Number of pooled suspensions tested.

TABLE 85

RESISTANCE OF ADULT RATS TO REINFECTION WITH AUJESZKY'S VIRUS

Primary infection			First challenge ($10^{4.5}$ $TCID_{50}$/0.5 ml.)				Second challenge ($10^{3.5}$ $TCID_{50}$/0.5 ml.)		
Dose of virus ($10^{TCID_{50}}$/0.5 ml.)	Responses	Number of survivors used	Responses		Number of survivors used		Responses		
			Infected mice	control mice	Infected mice	control mice	Infected mice (a)	Infected mice (b)	control mice
7.5	5/5								
6.5	5/5								
5.5	5/5								
4.5	4/5	1	0/1	2/5	1	3	1/1	3/3	6/6
3.5	4/5	1	0/1		1		1/1		
2.5	0/5	5	0/5		5		5/5		
1.5	0/5	5	2/5		3		3/3		
0.5	0/5	5	1/5		4		3/4		

Numerator = number died Denominator = number infected

a = sensitized mice that survived first challenge infection.

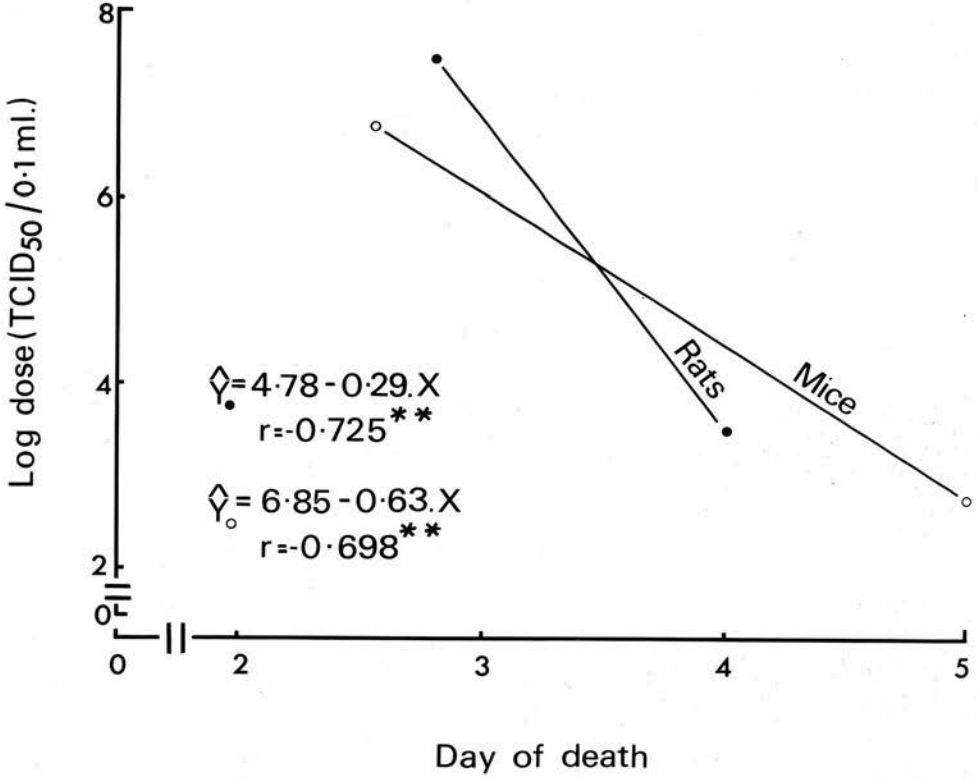
b = surviving control mice.

Fig. 72. - Conjunctivitis in a rat following intramuscular infection with the McFerran strain of Aujeszky's virus.

Fig. 73. - Reaction at the site of inoculation in a rat infected intramuscularly.

Fig. 74. - Orchitis and preputial discharge in a rat infected intramuscularly.

Fig. 75. - Comparison of the relationship between the dose of virus and the mean day of death in adult rats and mice infected intramuscularly with the McFerran strain of Aujeszky's virus.


Log dose (TCID50 / 0·1 ml.)
8
6
4
2
0
0
2
3
4
5
Day of death
Rats
Mice
Ŷ● = 4·78 - 0·29.X
r = -0·725**
Ŷ○ = 6·85 - 0·63.X
r = -0·698**

DISCUSSION

Pseudorabies virus is an unique member of the herpes group that is well known for its versatile spectrum of infectivity for cells derived from diverse hosts (Lautie, 1969; Kaplan, 1969); and the results of this present investigation are mostly in accord with those of other workers. However, contrary to the finding of Ivanovics et al., (1954) that suspension cultures of tissues derived from older chickens do not support the growth of the virus, the present studies showed clearly that monolayer cell cultures of kidneys obtained from chickens of different ages were susceptible to the infection. While there may be a number of reasons for this difference the discrepancy may be due merely to that fact that monolayers were used in place of suspended cell cultures; or to strain differences.

The cytopathological expression of the growth of the three strains of Aujeszky's virus was typical of the virus group and mostly consisted of rounding degeneration of individual infected cells or the early development of multinucleated polykaryons, according to the type of cell system used. Dissolution of the cytoplasmic boundaries which precedes or accompanies syncytial formation was especially marked in infected cultures of rabbit kidney cells and is probably a cytocidal attribute of the virus. Unfortunately, there is no clear evidence to date, to

show whether or not lysis of the cell membranes associated with the formation of syncytia in pseudorabies-infected cultures is due to the same "cell-fusion factors" that have been described in some paramyxovirus infections (Henle, Deinhardt and Girardi, 1954; Okada, 1958; Kohn, 1965). The function of the polykaryon in pseudorabies-infected cell cultures may be similar to that produced by measles virus which Norrby (1967) considered is a protective device directed towards reducing injuries to the cell membrane by the lytic enzymes located in the envelope of the virion. An interesting theory by Thomison (1962) that the formation of syncytia facilitates the progress of measles infection in the presence of specific neutralising antibody, could, if confirmed, help to explain the mechanisms of direct cell to cell transfer which is a feature of Aujeszky's and other herpes virus infections. The characteristic of rounding and clumping of cells in herpes simplex infections which have been attributed to the 'character' of the monolayer and to the age of the cells (Scott and McLeod, 1959) was also a distinctive feature of the cytopathology of pseudorabies infection in fowl embryo, chicken and mammalian cells of different ages.

That there was a negative correlation between the age of the PK 15 cells at the time of infection and the onset

of the cytopathic changes is of interest since a similar relationship has been reported for rinderpest virus in calf kidney cells (Plowright and Ferris, 1957), for tick-borne encephalitis virus in HeLa cells (Libikova and Smidova, 1962) and for measles and canine distemper virus in different types of cell cultures (Ramachandran, 1970); also Cheever and Wilmert (1942) observed that herpes simplex virus failed to replicate in suspension cultures of fowl embryo fragments more than four days old. It is well known that younger cells have a more active metabolism than older cells and it is evident from the studies of Levine, Becker, Boone and Eagle (1965) that RNA, DNA and protein synthesis in human diploid cells declines progressively with age. Another factor which may enable younger cells to induce an early CPE is their inability to produce large amounts of interferon compared with older cells (Carver and Marcus, 1967; Libikova, Rajcani and Henslova, 1969) which in turn, are more sensitive to the effects of interferon than are immature cells. However, the early development of CPE in young PK 15 cells following infection with Aujeszky's disease virus is probably not due solely to the quantitative aspects of interferon action since there is little evidence in the literature that interferon is produced by pseudorabies virus (Kaplan, 1969). Although there is a

report by Wawrzkiewicz (1966) of traces of interferon in cultures of calf and pig kidney cells and fowl embryo fibroblasts infected with various strains of Aujeszky's virus, that was active against Western Equine encephalomyelitis virus, the interferon produced had no inhibitory effect on pseudorabies virus. It is emphasised in discussing this present work, that the age of cultured cells had a significant effect on the onset of the cytopathic changes only, and not on the virus titres obtained.

The fact that fibroblasts and kidney cells from fowl embryos and chickens respectively of different ages were equally susceptible to infection with Aujeszky's disease virus suggests that cells derived from hosts at different metabolic and environmental states, behave in a similar manner when grown in vitro. On the other hand, the infectivity titres obtained in eggs were significantly higher when seven days old embryos were infected by the yolk sac route than for ten days old embryos inoculated on the CAM or allantois.

The value of electron microscopy and immunofluorescent staining for the rapid detection of virus and specific antigens in infected cell cultures was confirmed. The negative staining procedures were simple to carry out and both mature and immature virus particles showing the characteristic herpes-type morphology were readily

detectable in the culture fluids. Although the majority of virions were "naked" almost every field examined showed several enveloped virions and, on occasion, an envelope containing more than one virus particle. So far as could be ascertained the ultrastructure of the pseudorabies virion was identical to that of other members of the herpesvirus group.

Demonstration of viral antigens by immunofluorescence confers serological specificity to the diagnosis and the present findings showed that virus-specific antigens could be readily detected in PK 15 cultures as early as four hours after infection with a high dose of the McFerran strain of virus. These results were in agreement with those for herpes simplex virus (Lebrun, 1956), herpes simiae virus (Benda, 1965), varicella virus (Slotnik and Rosanoff, 1964), herpes zoster virus (Rapp and Vanderslice, 1964) and pseudorabies virus (Albrecht et al, 1963; Zuffa et al, 1968).

The replicative cycle of herpesviruses occurs mainly in the nucleus and Albrecht et al (1963), who studied the development of pseudorabies virus-specific antigens in fowl embryo cells, showed that the logarithmic phase of viral replication synchronised with the appearance of bright nuclear fluorescence. In the present study very few nuclei were seen with fluorescent particles in the

nuclear matrix and the exponential phase of virus development appeared to coincide with the appearance of a brilliant ring of fluorescence on the surface of the nuclear membrane. Later, as the infection proceeded, clearly defined areas of fluorescence developed in the perinuclear region and in the Golgi zone of the cytoplasm. Similar findings have been reported by Lebrun (1956) and Nii and Kamahora (1963) in herpes simplex infected FL and mouse fibroblast cultures respectively, and by Stewart, Carbrey and Knesse (1967), and Gustafson (1970) in pseudorabies-infected cultures, while the simultaneous appearance of specific herpes fluorescence in the nucleus and cytoplasm has been observed by other workers (Ross and Orlans, 1958; Munk and Fisher, 1965). These discrepancies in the distribution of herpes antigens in different cell systems are most probably due to differences in the reactivities of antibodies in the sera used and it is possible that the sera used in this present study contained antibodies that were only reactive with the envelope antigens which the virion acquires as it is extruded from the nucleoplasm of the infected cells. In general, the areas of an infected monolayer showing specific immunofluorescence were those that showed cytopathic changes when examined by conventional staining methods.

Advantage was taken of the fact that Aujeszky's disease virus contains DNA and replicates in the nucleus, to study its development in cell culture by means of acridine orange staining. The method, which is simple to perform, proved to be both rapid and reliable, and it is surprising that the technique is so little used in routine investigational work. Early changes in the nuclei of pseudorabies-infected cells and particularly those in the multinucleated syncytia which developed later, showed an abundance of brilliant DNA fluorescence. Much of these DNA is probably of viral origin because the sequential cytochemical studies of Ben-Porat and Kaplan (1965) have clearly shown that there is a progressive decrease in the rate of synthesis of cellular DNA soon after infection and which ceases completely by the seventh hour post-infection. At the same time, there is a concomitant increase in the intranuclear synthesis of viral DNA.

The detection of specific CF and ID antigens in pseudorabies-infected cell cultures was time-consuming because concentration procedures were necessary before reliable information could be obtained. Some of these antigens appeared to be soluble since no significant differences were detected in the relative concentrations of the CF and ID antigens obtained from infected cells or

cell culture fluids. Both types of antigens were heat stable and were not affected adversely when exposed to temperatures up to 75°C; a finding which is similar to that obtained by Tokumaru (1970) for the precipitating antigens of herpes simplex virus. It is of interest, on the other hand, that the CF antigens from herpes simplex infected cells are destroyed by heating for one hour at 56°C (Kaplan, 1969). The relationship of the soluble CF and ID antigens to mature virus is not clear but it has been postulated by Hamada and Kaplan (1965) and Kaplan (1969) that the soluble antigens of herpes simplex and pseudorabies virus are structural proteins which are incorporated into the mature virion at a late stage in the replicative cycle. Thus it is possible that the process of integration into the virion confers a degree of heat stability to the complement-fixing antigens of pseudorabies virus.

In the demonstration of neutralising antibodies the addition of guinea pig complement to the serum-virus mixtures enhances the neutralising capacity of human and rabbit sera (Yoshino and Taniguchi, 1964; Plowright, 1962; Muchel and Toussaint, 1962) but not bovine sera (Rweyemanu and Johnson, 1968). The potentiating effect was attributed to the 2nd, 3rd, and 4th components of the complement (Dozois, Wagner, Chemerda and Andrew, 1949).

In the present study, titres of neutralising antibodies to Aujeszky's virus in rabbit but not chicken sera were augmented by the addition of guinea pig complement, while rabbit complement produced an even higher titre. If antibodies in pig sera are proved to be susceptible to the enhancing effect of complement, the test incorporating guinea pig or rabbit complement is likely to be of value in differentiating past and recent outbreaks in pigs in endemic areas.

The results of thermal inactivation studies with the McFerran strain of Aujeszky's virus showed that it was stable at the temperatures commonly used in virus-neutralisation test. But, it is realised that temperature sensitive variants may also exist (Bodon et al, 1968). The literature contains little information on the half-life of pseudorabies virus at different temperatures, but in the present study, the estimated half-life of the virus at 37°, 22° and 4°C was 5.45, 12.50 and 88.25 hours respectively. These values are somewhat different from those quoted by Farnham and Newton (1959) and Scot, McLeod and Tokumaru (1961) for herpes simplex virus (1.5 - 3.0 hours at 37° and 3.75 - 13 hours at 30°C) but the value at 37°C approximates that for canine herpes-virus (Carmichael, Strandberg and Barnes, 1965).

A comparison of the sensitivities to Aujeszky's virus of cell cultures, fowl embryos, chickens, mice and rats shows that cell cultures and day-old mice are equally suitable for the isolation of the virus from suspect clinical material (Table 86). Also the susceptibility of fowl embryos to yolk-sac infection with Aujeszky's virus was significantly lower than that of PK 15 cells, but unweaned mice were apparently more susceptible than seven days old fowl embryos although the difference was not significant. The susceptibilities of day-old chicks and adult mice to intracerebral infection were of the same order and no significant differences were detected in the susceptibilities of adult rats and mice to intramuscular infection with the virus. While these findings confirm that cell cultures and day-old mice are eminently suitable for isolating and identifying Aujeszky's virus, they also clearly show that the fowl embryo is a sensitive indicator host, and one that should prove useful in small diagnostic laboratories where cell culture methods are not available. Day-old chicks and adult mice and adult rats could also be used, but are less sensitive.

The route of inoculation of the virus was found to have a profound influence on the nature, time of onset and severity of the clinical signs in rats and mice, and on

TABLE 86

COMPARISON OF THE SUSCEPTIBILITIES OF CELL CULTURE, FOWL EMBRYO, CHICKS, MICE AND RATS TO EXPERIMENTAL INFECTION WITH AUJESZKY'S VIRUS

Comparison	Difference in LD_{50}	S.E. of the difference	d.f.	t	P
PK15 cells and intramuscular infection of day-old mice	0.57	0.666	13	0.856	> 0.40
PK15 cells and yolk sac infection of fowl embryos	1.17	0.555	13	2.181*	< 0.05
Intramuscular infection of day-old mice and yolk sac infection	0.60	0.664	16	0.903	> 0.40
Yolk sac infection and intracerebral infection of day-old chicks	2.00	0.539	17	3.709**	< 0.01
Intramuscular infection of day-old mice and intracerebral infection of day-old chicks	2.60	0.570	17	4.561**	< 0.01
Intracerebral infection of day-old chicks and adult mice.	0.30	0.510	17	0.588	> 0.50
Intramuscular infection of adult rats and adult mice	0.70	0.383	27	1.827	> 0.05

the general mortality pattern in chickens as well as in rodents. The intracerebral route was the most effective and the mean incubation periods in mice and rats were very similar to the figures cited for intracerebrally infected laboratory-bred mice and hamsters (Reagan et al, 1952); but lower than those for rabbits (Hurst, 1934; McFerran and Dow, 1962).

The effect of the route of inoculation on the clinical parameters of experimental Aujeszky's disease in farm animals is the subject of several reports, but there can be little doubt that intracerebral infection is the most effective in all animal species. The effects of administering the virus by other routes are less definite and variable results have been reported in pigs by Shahan et al (1947a); McFerran and Dow, (1965) and Olander et al (1966) and in cattle and sheep by McFerran and Dow (1965). No significant differences were observed in the mean incubation periods in intracerebrally infected rats and mice but differences did occur in the time of onset of clinical symptoms following other methods of inoculations (Table 87).

TABLE 87

COMPARISON OF THE MEAN INCUBATION PERIODS (HOURS) IN EXPERIMENTAL AUJESZKY'S DISEASE OF MICE AND RATS

Route	Mice	Rats	S.E. of the difference	d.f.	t	P
Intracerebral	28	30	4.72	12	0.487	> 0.50
Intravenous	36	53	8.33	13	2.761**	< 0.02
Intraperitoneal	48	42	2.95	13	2.059	> 0.05
Intramuscular	47	39	3.18	13	2.373*	< 0.05
Intranasal	61	42	7.62	17	2.495*	< 0.05
Intraocular	83	44	7.84	21	4.913**	< 0.01

For instance, the incubation periods following intramuscular, intranasal or intraocular infection were significantly shorter in rats than in mice. The differences following intraocular inoculation are probably related to the wider dissemination of the virus in the internal organs of rats than in mice (Tables 75 and 82). It is possible that the differences in the mean incubation periods in intranasal and intramuscular infections of rats and mice are due to differences either in the mechanisms of dissemination of the virus or in the rates of uptake of the virus from the sites of inoculation. The pathways by which herpes simplex and pseudorabies virus reach the central nervous system in experimentally

infected mice were carefully investigated by Johnson (1964a) and Sabin (1938), respectively. In view of their findings, it seems that the differences observed in this present study in the mean time of onset of clinical symptoms and in the mean duration of infection in intramuscularly or intranasally infected mice and rats are mainly due to innate differences in the mechanisms and rate of spread of the virus in the two hosts (Table 88).

TABLE 88

COMPARISON OF THE MEAN PERIODS (HOURS) OF FATAL INFECTION IN EXPERIMENTAL AUJESZKY'S DISEASE OF MICE AND RATS.

Route	Mice	Rats	S.E. of the difference	d.f.	t	P
Intracerebral	40	41	0.863	12	0.927	> 0.30
Intravenous	53	70	10.390	13	1.508	> 0.10
Intraperitoneal	63	66	2.760	13	1.254	> 0.20
Intramuscular	59	60	2.750	13	0.359	> 0.70
Intranasal	66	71	18.750	17	0.267	> 0.80
Intraocular	91	64	5.600	21	4.766**	< 0.01

Although the findings might also suggest that rats are the more sensitive indicator hosts to Aujeszky's virus, the differences in the LD_{50} values for the virus

in intramuscularly infected rats and mice were not significant (Tables 74 and 81; $t = 1.827$; $P > 0.05$).

An interesting and significant finding was that intravenously infected mice became clinically ill before similarly infected rats (Table 87). However, the difference may be due merely to the fact that the same dose of cell culture virus contained relatively more infective units for mice than for rats because of their smaller blood volume and body weight. It is also of interest that the virological data shown in Tables 75 and 82 indicates that in intravenously infected mice virus was distributed to all of the internal organs examined clearly establishing a larger number of primary infective foci than in experimentally infected rats.

The mortality, duration and clinical signs of the disease in day-old chicks were reminiscent of the disease in rodents except that hyperaesthesia of the skin was not observed. Moreover, as in the rats and mice, the mortality rates in chickens were dose-dependent and were also significantly related to the route of infection. It is emphasised that the percentage of mortalities in groups of chickens of different ages reported in the present study was far higher than the figures mentioned in the reports of Bang (1942) and Ivanovics et al (1954).

Much of the contradiction in the early literature on the susceptibility of poultry to experimental infection undoubtedly arises from the fact that birds of undetermined and possibly older age-groups were generally used. The results of the present study have clearly established the decisive influence of age on the susceptibility of chickens and mice to fatal infection with the virus. Age-related resistance to viral infections of man and animal is a well-documented phenomenon (Sigel, 1952; Burnet, 1960). Much of the early experimental work attributed the development of this age-associated resistance to viral infections to the presence of 'barriers' located in the anterior region of the brain, and on the 'end plates' of voluntary muscles (Sabin and Olitsky, 1937a and 1937b; 1938a). Later work (Sabin and Olitsky, 1938b; 1941) suggested that the "age barriers" might be located at the level of blood vessels, but the nature of cells constituting the 'barriers' was not understood until the importance of macrophages in the pathogenesis of viral infections was described (Brunner, Hurez, McCluskey and Benacerraf, 1960; Kantoch, Warwick and Bang, 1963; Mims, 1964). In his studies of herpes simplex encephalitis in unweaned mice, Johnson (1964b) postulated that an alteration in the macrophages of the maturing mouse plays an important role in the development

of resistance to herpesvirus encephalitis. In vitro, infected adult mouse macrophages failed to infect other cells whereas, the sucking mouse macrophages transferred the infection to adjacent cells in the culture and also to a variety of added cells. It is conceivable that resistance to intramuscular infection to pseudorabies virus in chicks with increasing age is related not only to the presence of 'barriers' at the myoneural junctions but also to alterations in the free macrophages or fixed mesenchymal cells in the tissues. However, macrophages did not seem to have a significant role in the studies of Subrahmanyan (1968) on the susceptibility of mice of different ages to poxvirus.

The literature shows that spread of Aujeszky's virus in the body differs considerably in different species of animals. In the present study, the virus was readily isolated from the brains of all chickens, mice and rats dying of the disease, including those that were infected peripherally with minimal amounts of virus. Isolation of virus from other tissues largely depended on the route of infection, the animal species, and the strain of virus used. For instance, intracerebral or intra-ocular infection of rats resulted in the dissemination of virus in the heart, lung and some times the adrenal gland, in addition to the brain, but the virus was not recovered from extra-neural tissues in intracerebrally

infected mice; the latter finding being reminiscent of that reported in herpes simplex infections of sucking mice (Johnson, 1964a). Virus was not recovered from the liver and spleen of mice infected by the intraperitoneal route (Table 75) although it was present in high titres in these organs, as well as in the adrenal glands, of experimentally infected rats. Intraperitoneal infection in rats appeared to result in the spread of virus only to the abdominal viscera (Table 82). In general, the patterns of virus distribution in rats and mice following most peripheral routes of infection suggests that viraemia has an insignificant role in the pathogenesis of the infection. The fact that the virus was recovered from the kidneys of rats but was not detected in the urine, suggests that viral replication had taken place in the nervous rather than in the epithelial tissue.

Virus distribution in infected chicks merits comment. Chicks of one to hundred and eighty days of age infected intracerebrally contained virus only in the brain and in no other tissue, and the rate of virus replication, but not the virus titres, in the brain were related to the age of chick at the time of infection. Likewise, the extent of the viraemia following intracerebral or intramuscular infection and the replication of virus in the muscle tissue at the sites of inoculation were significantly related to age. The Hungarian strain of Aujeszky's

virus, unlike the McFerran strain, established itself with ease in the different tissues after intracerebral or intramuscular inoculation. Virus titres in the muscle tissue were higher than those in the brain and the LD_{50} values of the virus were significantly higher in the intramuscular route than in the intracerebral route. The difference in the affinity of the Hungarian strain for the extra-neural tissues may be a genetic attribute. Following its isolation by the plaque technique the strain was avirulent for pigs, calves, sheep and dogs; and infected rabbits did not show pruritus (Kojnok, 1965). The strain also underwent several serial passages in fowl embryo cells (Skoda, 1962) which might have diversified its tissue tropism.

A comparison of the distribution of Aujeszky's virus in mice and rats described in the present study with the findings of McFerran and Dow (1964a, 1964b, 1965) and Dow and McFerran (1966) for pigs, calves and sheep shows that pig and rodents are potentially more important than ruminants as reservoirs of the infection. In pigs, mice and rats infected intranasally virus was isolated from the brain and lung and also occasionally from spleen and kidney. The virological and immunocytological observations of Sabo et al (1968) and Rajcani et al(1969) showed similar distribution of virus and/or viral antigens in perorally or subcutaneously infected pigs. The

possible role of mice in the spread of Aujeszky's disease is illustrated by the fact that lateral transmission of the virus occurred between infected litters and their dams and that infection was probably acquired either by inhalation or by injection or by both routes. A similar mode of spread between the infected progeny and mothers is highly likely in rats, although contact infection in adult rats was not observed under experimental conditions (McFerran and Dow, 1970). Even if rats and mice do not serve as an important reservoir host of Aujeszky's disease virus, it cannot be excluded that during the incubation period, rats may play a role in the spread of the disease from farm to farm.

The epizootiological role of domestic poultry in the spread of Aujeszky's disease is a matter for speculation. The findings in the present study suggest that very young chicks are susceptible to experimental infection by different parenteral routes, including the peroral, nasal and the dermal. It is possible that in infected premises young chick may acquire the infection without showing classical symptoms of the disease but they are unlikely to act as reservoir hosts or transmit infection to other susceptible farmstock. There was no evidence of excretion of virus in the natural discharges of infected chicks; but these findings were based on the

behaviour of the McFerran strain of virus that has been propagated in mammalian cell cultures. It is possible that other strains might behave differently. For instance, viraemia with Aujeszky's strain of virus occurred more regularly than with Iowa strain of virus (Shope, 1931). Whereas Ivanovics et al (1954) induced fatal subcutaneous infection in chicks up to 16 days of age with two isolates of the virus, in the present study, subcutaneous infection with the McFerran strain was fatal to chicks up to 60 hours of age only. Moreover, in the present study, virus dissemination into several organs of infected day-old chicks was a predominant feature with the Hungarian strain, whereas virus was demonstrable only in limited tissues in chicks infected with the McFerran strain. However, rodents preying on infected young chicks might acquire the infection and thus perpetuate the cycle of spread postulated by Shope (1931).

Many of the chicks and a few mice and rats that survived primary infection were resistant to reinfection. The ability to withstand reinfection by the intracerebral route in very young chicks was significantly related to the dose of virus in the first inoculum. Survival of chicks of older age-groups following challenge infection was likewise conditioned by the degree of first exposure to the virus and was independent of age-associated tolerance. The fact that a few mice survived two

successive challenges at fortnightly intervals suggests that during a natural outbreak of the disease a number of infected mice, and possibly other species of rodents also, may recover from the clinical disease and act as carriers of the infection.

In the present study, the serological response of the chicken to experimental infection with Aujeszky's virus was of a low order and was comparable with the magnitude of the response reported in pigs recovered from natural disease (Shope, 1935a; Berbinschi, 1956; Johnston et al, 1961; Akkermans, 1963; Skoda et al, 1963) or experimental infection with live vaccine (Kojnok, 1963; Sabo, 1969). Although the titres of neutralising antibodies in pigs receiving 2 doses of the vaccine were significantly higher than those inoculated with a single dose (Kojnok, 1963; Zuffa, 1963b; 1964), there was no relationship between the levels of neutralising antibodies and the ability to resist challenge infection. Likewise, in the studies of Kojnok (1963), cattle, sheep and dogs, immunised with one to five ml. of one dose of live vaccine (Bartha strain) withstood intramuscular challenge infection with virulent virus, despite the fact that the neutralising antibody titres in these animals were poor or not detectable.

In pseudorabies, resistance to a further exposure of

virus is probably not mediated solely by neutralising antibodies. The literature on the pattern of the disease in pigs, ruminants, carnivores, and rodents makes it clear that the virus travels along the nerve tracts to exert its virulence in the central nervous system, and perhaps secondarily along the lymphatics. Since viraemia is, at best, transient or of a low order, the application of antiserum is not likely to be effective in arresting viral invasion of the central nervous system, although in vaccinated animals circulating neutralising antibodies might neutralise the virus released into the tissues or in the body fluids. The latter possibility finds support in the studies of McFerran and Dow, (1965) and Sabo, (1969), in which the disappearance of the virus from infected animals coincided with the onset of neutralising antibodies. Nevertheless, Sabo showed that virulent virus continued to be released in the pharyngeal secretions for 18 and 20 days after peroral infection of susceptible and immunised piglets respectively, a period long enough to ensure dissemination of the virulent virus in the susceptible stock in the herd. But, unlike in pigs in which a carrier-state has been proven (Kojnok, 1965; Wilke and Dannenberg, 1968) there is no evidence in the present study of excretion of virus in the chicks infected with the McFerran strain. The development of

neutralising antibodies in low titres in experimentally infected chickens is also consistent with the transient viraemia.

CONCLUSION

Aujeszky's virus exists in reservoir hosts and transfer to natural hosts is mediated by mechanisms that are imperfectly understood. The virus has a special relationship with some of its natural hosts in which selective adaptation appears to have culminated in unique synergism. Its encounter with the host is manifest as overt disease with spectacular symptoms or results in subclinical, inapparent or latent infection.

In reservoir hosts such as the pig and rat, a host-to-host cycle is perpetuated by transfer of infectious virus through the upper respiratory or the oro-pharyngeal routes but the cellular and molecular basis of the stabilised host-parasite relationship which permits carriage of the virus in the host tissues has not been elucidated, although cell culture studies suggest that the virus can spread from cell to cell in the presence of virus-neutralising antibodies. In the so-called "dead-end" hosts, such as ruminants and carnivores, the virus-host encounter is almost always fatal to the host.

The nature of the relationships of Aujeszky's virus

with its several hosts is so varied that no single laboratory model can be expected to provide a meaningful basis of the different ecological aspects of the association. In the present study, the responses of *in vitro* and *in vivo* systems to infection with strains of Aujeszky's virus were assessed on a comparative basis with the object of correlating the findings with those established in other experimental systems. It is clear that the virus is readily isolated in cell cultures, fowl embryos, young chicks and laboratory-bred rodents and is readily demonstrable in infected cells by electron microscopy, fluorescent antibody and traditional cytological techniques. The use of potent sera in conjunction with fractionation of infected tissues or tissue culture fluids might facilitate the demonstration of virus-specific antigens. Investigations of the chemistry and reactivities of these antigens are necessary for the understanding of the intimate relationship of the virus with the host cell types and the relationships that exist between pseudorabies and other herpesviruses. Finally, studies of the pathogenesis of the virus following different routes of infection in reservoir hosts, and potentially susceptible hosts such as the young chick might elucidate the means by which the virus spreads within these hosts and the nature and magnitude of the factors associated with specific and non-specific resistance.

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PUBLICATIONS

STUDIES ON THE VIRUS OF AUJESZKY'S DISEASE

I. Pathogenicity for Rats and Mice

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INTRODUCTION

The means by which Aujeszky's disease is transmitted under natural conditions is not fully understood.

Swine are generally regarded as one of the natural reservoirs of the virus (Galloway, 1938). Other workers believe that wild rodents may play an important role in transmitting the infection and Balas (1908) postulated that dogs and cats may contract the disease by feeding on infected rats. Hutyra (1910) found several infected rats on farms where Aujeszky's disease was present amongst cattle, and was able to demonstrate the presence of the virus in their brains. Shope (1935a, 1935b), who observed that pigs became infected by eating the carcases of infected rats, postulated a cycle of infection from rats to pigs, thence to cattle and back to rats. There is also a report by Gerlach and Schweinburg (1936) that Aujeszky's disease can be transmitted experimentally to rats and guinea-pigs by the bites of infected rats and, more recently, Cassells and Lamont (1942) and Lamont and Gordon (1950) described the disease in rat-terriers. Lamont (1947) also showed that rats are comparatively easily infected by subcutaneous inoculation or by feeding them on portions of the carcases of rabbits dying from the disease.

The possibility that rats may play an important role in transmitting the infection has also been emphasised by Lamont (1947) and Ledyaev and Rakhmanov (1964) who found disease followed by deaths in rats on farms shortly before the occurrence of the disease in young cattle and pigs, respectively; likewise Janowski and Oberfeld (1965) noted that rats disappeared suddenly from a piggery during an outbreak of the disease in swine.

Although there is circumstantial evidence to show that the rat may act as a minor reservoir of the virus, there appears to be very little direct experimental proof of this. Information is also lacking on a number of other epidemiological aspects of the disease in rats, particularly the pathogenesis and the factors affecting lateral transmission of the virus in rodents. This study of the pathogenicity of the virus of Aujeszky's disease for laboratory rats and mice was undertaken in an attempt to answer some of these questions.

MATERIALS AND METHODS

Virus. The virus was originally isolated in rabbit tissue culture from a case of Aujeszky's disease in a pig. The preparation used had a titre of $10^{6\cdot5}$ 50 per cent. tissue culture doses ($TCID_{50}$) per 0·5 ml. (McFerran and Dow, 1965). In the present investigation all studies were carried out with a pool of virus grown on a continuous line of pig kidney cells and stored at −65°C. The titre ranged from $10^{6\cdot8}$ to $10^{8\cdot1}$

$TCID_{50}$ per 0·2 ml. In one experiment the virus was propagated on a secondary cell culture of puppy kidney. Titres were calculated according to the method of Reed and Muench (1938) and the procedure for determining the approximate error of LD_{50} was that of Pizzi (1950).

Tissue culture. The puppy kidney culture and the continuous line of pig kidney cells (PK15) were grown in Earle's balanced salt solution containing 0·5 per cent. lactalbumin hydrolysate, 0·1 per cent. yeast extract and 10 per cent. heat-inactivated calf serum or in Eagle's 59 medium containing 10 per cent. tryptose-phosphate broth and 10 per cent. inactivated calf serum. For maintaining the cells, the same media were used but with calf serum reduced to 2 per cent. Two hundred units of penicillin, 100 μg. of streptomycin and 25 units of nystatin per millilitre were incorporated in all media.

Animals. The rats were a laboratory bred stock of the CFE strain and were used at 10 weeks of age and approximately 250 g. body weight. The mice were from a laboratory bred stock of mixed population and were used as adults (8 to 13 weeks), week-olds or day-olds.

Virus isolations. Portions of brain, heart, lung, spleen, liver and kidney and, in some instances thigh muscle, were taken at post-mortem and ground up in sterile sand by pestle and mortar or in a small glass Griffith's tube. Maintenance medium containing antibiotics was added as the diluent to give an approximate 10 per cent. suspension. After light centrifugation, four tissue culture tubes per specimen were inoculated with the supernatant fluid, incubated on a roller-drum at 37°C. and examined daily for seven days. The virus was detected by its characteristic herpes virus-type cytopathic effect and identified, when necessary, by neutralisation with specific antiserum. In cases where no cytopathic effect was detected, at least three further passages were carried out before the specimen was regarded as being negative.

RESULTS

Susceptibility

The disease pattern of experimental Aujeszky's disease was investigated in small groups of adult mice that were infected by various routes. The virus had a titre of $10^{6\cdot8}$ $TCID_{50}$ per 0·2 ml. and was used in a dilution of 10^{-1}. The results of this preliminary experiment showed that laboratory mice are susceptible to infection with the virus, irrespective of the route of inoculation, and that the great majority died after a brief illness (Table 1). During this and later experiments it was quite exceptional for an animal to survive after showing clinical signs of the disease. The incubation period ranged from 24 to 120 hours, depending on the route of infection. It was longest in mice inoculated intranasally or intraocularly and some animals required a second injection to initiate the infection. The distribution of the virus in tissues taken at post-mortem showed that virus was present in the brains of all mice dying from the disease. Virus was present in the brains but in no other tissue of mice inoculated intracerebrally or intraocularly, whereas in mice infected by various other routes, the virus was distributed irregularly in the tissues. The spleen was infected only after intravenous inoculation whereas intramuscular inoculation did not lead to infection of the heart, lung or spleen. Virus was isolated from the kidneys of all mice inoculated by the intramuscular, intravenous and intraperitoneal routes and a few that were inoculated intranasally.

In a similar experiment in adult rats, using 10 animals per group, the virus produced almost 100 per cent. mortality within three days, irrespective of the route of infection. The single exception was a rat that required a second inoculation of virus before infection was established. The distribution of the virus in

rats was investigated by the intramuscular route only and the virus was isolated from the site of inoculation, the brain and the kidneys; but from no other tissue.

A comparison was made of the susceptibility to Aujeszky's disease virus of two types of tissue culture and adult mice inoculated by various routes and virus titres ($TCID_{50}$ and LD_{50}, respectively) were obtained as follows: continuous pig

TABLE 1
MORTALITY, DURATION OF INFECTION AND DISTRIBUTION OF VIRUS IN ADULT MICE INOCULATED BY VARIOUS ROUTES

	Virus (ml.) and route of inoculation					
	Intra cerebral (0·05)	*Intra muscular (0·5)*	*Intra venous (0·5)*	*Intra peritoneal (0·5)*	*Intra nasal (0·3)*	*Intra ocular (0·3)*
Mortality	8/8 (100%)	10/10 (100%)	8/8 (100%)	13/13 (100%)	14/16 (88%)	5/8 (63%)
Incubation period (hr.)						
Range	24–40	40–53	24–60	40–53	24–78	64–120
Mean	(28)	(47)	(36)	(48)	(61)	(83)
Duration of infection (hr.)						
Range	40	52–62	48–60	60–72	52–78	76–120
Mean	(40)	(59)	(53)	(63)	(66)	(91)
Distribution of virus						
Brain	+	+	+	+	+	+
Heart	−	−	+	+	+	−
Lung	−	−	+	+	+	−
Liver	−	−	+	−	−	−
Spleen	−	−	+	−	−	−
Kidney	−	+	+	+	−	−
					(∓)	

+ = virus isolated − = virus not isolated (∓) = virus very occasionally isolated
Mortality Numerator = number dead. Denominator = number inoculated

kidney cell line PK15 ($10^{7\cdot1}$); secondary dog kidney cell culture ($10^{6\cdot8}$); adult mice, intracerebral ($10^{4\cdot5}$), intramuscular ($10^{4\cdot5}$), intraperitoneal ($10^{4\cdot2}$) and intravenous ($10^{3\cdot1}$). Thus adult mice are appreciably less susceptible to the virus of Aujeszky's disease than are the two tissue culture systems.

Clinical Syndrome

Most mice inoculated intracerebrally were recumbent and comatose by the second day and died in less than 48 hours. Early symptoms of the disease were also produced by intravenous injection and most affected animals showed a mild, intermittent but generalised pruritus that was accompanied by frequent "face-washing" movements of the fore-legs. The pruritus and oedema in the head region were much more pronounced after 40 hours and the sick animals frequently collapsed on one side in an exhausted state. Similar signs were seen in mice infected by the intraperitoneal route. Mice that were successfully infected by instilling virus into the anterior nares usually became very ill by the end of the second day, but some required a second inoculation before becoming infected. Although there was no evidence of skin irritation the affected animals lay curled up and appeared to be quite blind in both eyes and a few showed violent, spasmodic movements an hour or so before they died. The earliest signs of infection

in mice inoculated intramuscularly appeared within 48 hours when the affected mouse started to gnaw or scratch the site of inoculation. After a few hours the pruritus became so severe that the animal would chew furiously and continuously at the wound until it collapsed and died. Virus fluid instilled into the conjunctival sac produced a severe reaction in the periorbital region and on the skin of the face, following a somewhat prolonged incubation period. Oedema of the head and intense pruritus were not apparent until the third, fourth or even fifth day when the mouse became greatly distressed and scratched and tore furiously at the skin of its face with its hind legs until death supervened.

In general, the clinical syndrome in rats was similar to that in mice except that the lesions in rats were more pronounced and more extensive. Rats inoculated intramuscularly in the hind leg invariably showed an inflammatory reaction around the eyes and a wide patch of oedema of the skin surrounding the point of inoculation. The fur, within the well defined borders of the oedematous area, was moist, discoloured and matted. Many of the rats were affected with diarrhoea and male animals frequently showed orchitis and a profuse sticky-type of discharge from the preputial orifice. There were, however, no obvious abnormalities of the external genitalia of female rats.

TABLE 2
MORTALITY PATTERN IN RATS AND MICE INJECTED INTRAMUSCULARLY *

	Rats	*Mice*										
Dilution of virus (log)	*Mortality*	*Mortality*	*Days to death*									
	No.	*No.*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
—1	5/5 (100%)	6/6 (100%)	–	6	–	–	–	–	–	–	–	–
—2	5/5 (100%)	18/18 (100%)	–	–	8	10	–	–	–	–	–	–
—3	5/5 (100%)	37/38 (97%)	–	–	7	30	–	–	–	–	–	–
—4	4/5 (80%)	32/39 (82%)	–	–	6	15	9	1	1	–	–	–
—5	4/5 (80%)	11/37 (30%)	–	–	–	4	2	2	3	–	–	–
–6	0/5 (–)	0/24 (–)	–	–	–	–	–	–	–	–	–	–
—7	0/5 (–)	0/6 (–)	–	–	–	–	–	–	–	–	–	–
LD_{50}	–5·3	–4·6										

Numerator = number dead. Denominator = number inoculated
* $TCID_{50}$ was $10^{8\cdot1}$ per 0·2 ml.

Mortality Pattern

The mortality pattern in adult rats and mice inoculated intramuscularly with a high titred virus ($10^{8\cdot1}$ $TCID_{50}$ per 0·2 ml.) is set out in Table 2. The results show that the duration of the infection in mice inoculated by the intramuscular route is dose dependent. Although the difference in the LD_{50} values for rats and mice is small, there is some evidence that the rats were more susceptible to Aujeszky's disease virus since the values, with their standard errors, for the LD_{50} were $-5\cdot3$ ($\pm0\cdot395$) for rats and $-4\cdot6$ ($\pm0\cdot155$) for mice ($P < 0\cdot05$).

TABLE 3

MORTALITY PATTERN IN MICE OF DIFFERENT AGE GROUPS INJECTED INTRAMUSCULARLY

Age	Diln. of virus (log)	Animals used: Dam	Animals used: Number in litter	Days and number of deaths: 1	2	3	4	5	6	7	8	9	Mortality	D.I. (hr.)	Number of unweaned mice eaten by dams
Day-old	—1	A	8	—	8	A*	—	—	—	—	—	—	8/8	45	1
	—3	B	13	—	11	2	—	B*	—	—	—	—	13/13	51	2
	—5	C	9	—	—	9	—	—	—	C*	—	—	9/9	72	9
	—7	D	6	—	—	3	1	1	—	—	—	D*	5/6	86	4
	—9	E	9	—	—	—	—	—	—	—	—	—	0/9	—	0
													$LD_{50}^{-7.78}$		
Week-old	—1	F	6	—	6	—	—	—	—	—	—	—	6/6	47	0
	—3	G	10	—	10	—	—	—	—	—	—	—	10/10	54	0
	—5	H	11	—	3	8	—	—	H*	—	—	—	11/11	65	6
	—7	I	7	—	1	1	2	—	—	I*	—	—	4/7	80	1
	—9	J	10	—	—	—	—	—	—	—	—	—	0/10	—	0
													$LD_{50}^{-7.24}$		
Adult	—1		6	—	6	—	—	—	—	—	—	—	6/6	57	
	—3		6	—	3	2	—	—	—	—	—	—	5/6	85	
	—5		6	—	—	3	—	—	—	—	—	—	3/6	99	
	—7		6	—	—	—	—	—	—	—	—	—	0/6	—	
	—9		6	—	—	—	—	—	—	—	—	—	0/6	—	
													$LD_{50}^{-4.7}$		

D.I. = Mean duration of infection in hours of mice dying from the disease.
* = Death of the dam. Numerator = number dead. Denominator = number inoculated.

Effect of Age on Susceptibility

The effect of the age of the animal and the dose of virus on the duration of infection was investigated in groups of adult, week-old and day-old mice, all of which were inoculated intramuscularly with the same amount of virus. The results of this experiment show that the values, with their standard errors, for the LD_{50} were $-4{\cdot}7$ ($\pm 0{\cdot}767$) for the adult mice, $-7{\cdot}24$ ($\pm 0{\cdot}587$) for the week-old mice and $-7{\cdot}78$ ($\pm 0{\cdot}461$) for the day-old mice (Table 3). The values for the latter groups were greater ($P < 0{\cdot}01$) than that for the adult group, indicating that susceptibility of mice to Aujeszky's disease virus decreases with age. Although there was a suggestion that one-week old mice were more resistant than day-old mice the difference in LD_{50} between the two groups was not significant.

Lateral Transmission of Infection

It was interesting to note that a number of dams of litters infected with virus died during the course of the above experiment (Table 3) and that each of them was known to have eaten some of the infected carcases in her litter. On the other hand, the dams that had not eaten infected carcases as well as the dams of litters that survived the infection survived. The virus was recovered from the brain, heart, lungs and spleen, but not kidneys of the dead mothers, and from the brain and kidneys only of the baby mice.

Resistance

Attempts were made to demonstrate resistance in adult rats and mice that had survived exposure to the virus of Aujeszsky's disease. In the first experiment the 12 surviving rats from a previous trial were challenged intramuscularly after three weeks with 0·5 ml. of a 10^{-2} dilution of virus, but all succumbed within three days (Table 2).

In the second experiment adult mice, which are somewhat less susceptible than rats, (vide supra) were inoculated with the same pool of virus but in ten-fold dilutions from 10^{-3} to 10^{-6} (Table 4). The survivors, if any, from each of these inoculations were divided into three groups and were challenged fifteen days later with the same virus in dilutions of 10^{-2}, 10^{-3} and 10^{-4}, respectively. At the same time four groups of control mice were inoculated with dilutions of 10^{-2} to 10^{-5}, respectively. The survivors from this experiment and six controls were then reinoculated fourteen days later with a 10^{-4} dilution. The few mice that survived this challenge were inoculated along with controls thirteen days afterwards with a 10^{-2} dilution and, finally, the survivors were challenged a fortnight later with undiluted virus. The results show that of 47 adult mice that were not affected clinically by the higher dilutions (10^{-4} to 10^{-6}) of the primary inoculum, 14 survived the first challenge with 10^{-3} or 10^{-4} dilutions of virus, which are equivalent to 40 and 4 LD_{50} doses, respectively. Nevertheless, when these animals received a second challenge as many as 12 (86 per cent.) of the 14 mice survived compared with only 6 (24 per cent.) of the 25 susceptible mice that received the same amount of virus (10^{-4}) in the primary inoculation and none of the six control mice survived although these were of the same age and received the same inoculum as the experimental group. When the 12 survivors were challenged for the third

TABLE 4

RESISTANCE OF ADULT MICE TO SUCCESSIVE FORTNIGHTLY DOSES OF VIRUS

Primary inoculation			*First challenge*			*Second challenge*		*Third challenge*		*Fourth Challenge*	
Dilution of virus (log)	*Mortality*	*Number of survivors used*	*Dilution of virus*			*Number of survivors used*	*Virus 10^{-4}*	*Number of survivors used*	*Virus 10^{-2}*	*Number of survivors used*	*Undiluted virus*
			10^{-2}	10^{-3}	10^{-4}						
—3	25/25	—	—	—	—	—	—	—	—	—	—
—4	19/25	6	2/2	1/2	0/2	3	1/3	2	2/2	—	—
—5	6/25	17	5/5	6/6	1/6	5	0/5	5	3/5	2	2/2
—6	0/25	24	8/8	7/8	3/8	6	1/6	5	4/5	1	1/1
Total survivors used		47				14		12		3	
		Mortality	100%	88%	25%		14%		75%		100%
		Controls	10^{-2}		6/6						
			10^{-3}		7/7						
			10^{-4}		7/8	1	1/1				
			10^{-5}		2/6	4	4/4				
					Controls	10^{-4}	6/6 Controls	10^{-2}	5/5		

Numerator = number dead Denominator = number inoculated

time with one hundred times the amount of virus (approximately 400 LD_{50}) administered on the previous occasion, 3 (25 per cent.) survived compared with 100 per cent. mortality in the control group. Thus, these three mice had withstood at least three intramuscular injections of lethal amounts of the virus without showing clinical signs of the illness. Nothing is known about the nature or the duration of this resistance since the three mice ultimately succumbed to a final challenge with undiluted infected tissue culture fluid representing at least 40,000 LD_{50} doses of virus.

DISCUSSION

McFerran and Dow (1962) showed that there was no significant difference in the susceptibility to Aujeszky's disease virus between rabbits inoculated intracerebrally or subcutaneously and secondary cultures of pig or rabbit kidney cells. In the present work the McFerran strain gave appreciably lower titres in adult rats ($10^{5\cdot3}$) and adult mice ($10^{4\cdot6}$) than on a continuous line of pig kidney cells ($>10^{6\cdot8}$), and it is likely, therefore, that adult rats and mice are less susceptible than rabbits. On the other hand, unweaned mice were particularly susceptible, the virus titres in week-old and day-old mice being $10^{7\cdot25}$ and $10^{7\cdot8}$, respectively. Unweaned mice are, therefore, probably just as suitable as rabbits and tissue cultures for the isolation and identification of the virus of Aujeszky's disease.

The literature shows that the spread of Aujeszky's disease virus in the body differs considerably in different animal species. In mice, the virus was readily isolated from the brains of all that died from the disease including those that were inoculated with minimal amounts of virus into the peripheral parts of the body. Recovery of the virus from other tissues largely depended on the route of inoculation.

It is interesting to compare the distribution of the virus in mice with the findings of McFerran and Dow (1964a, 1964b, 1965) and Dow and McFerran (1966) for pigs, calves and sheep since it is generally believed that rodents and pigs may play an important role in the transmission of Aujeszky's disease whereas calves and sheep are considered unlikely to act as reservoir hosts of the virus. In sheep and calves dying from the disease following intramuscular inoculation of the virus into the hindquarters the virus is usually found in the sacral, lumbar and thoracic cord, but not in the brain or any extra-neural tissues whereas in mice, a less susceptible species, the virus can be isolated from the brain and kidney. Although the urinary bladder was not examined the presence of the virus in the kidneys of infected mice is of interest since the part played by the urine of rodents and pigs in the spread of Aujeszky's disease is still controversial. Intranasal infection of calves and sheep resulted in death associated with the presence of the virus in the brain, but in no other organ whereas in pigs and mice inoculated intranasally the virus could be isolated from the brain and lung and, occasionally, from the spleen and kidney. These observations suggest that pigs and mice are potentially more important than ruminants as reservoirs of the infection. The possible role of mice in the spread of Aujeszky's disease is further emphasised by the fact that lateral transmission of the virus occurred between infected litters and their dams, and that the infection was probably acquired by either inhalation, ingestion or by both routes.

Recovered pigs usually show neutralising antibodies in their sera, but they may, nevertheless, succumb to a second challenge of the virus if this is inoculated by the intracerebral route. It is also kown that rabbits and guinea-pigs which have resisted a lethal dose of virus given subcutaneously will succumb to intracerebral challenge. On one occasion Shope (1931) observed a guinea-pig which became immune to Aujeszky's disease and was subsequently inoculated eight times subcutaneously with virus without showing evidence of illness. During the present investigation, variations in individual susceptibilities to the virus of Aujeszky's disease were demonstrated by the survival of a few mice which showed early clinical symptoms of the disease before they succumbed to a subsequent infection. The fact that three mice survived two successive challenges at fortnightly intervals with approximately 4 LD_{50} doses of virus and even withstood a single test dose of 400 LD_{50} suggests that during a natural outbreak of the disease a number of infected mice and, perhaps, other species of wild rodents may recover from the clinical disease and act as carriers of the infection. Such a situation could be potentially dangerous not only for highly susceptible baby mice but also for pigs, dogs, cats and other animals that might eat infected rat or mouse carcases or be bitten by rats. These observations would also suggest that in countries where Aujeszky's disease in endemic, wild rats and mice are likely to be less susceptible, but potentially more dangerous to, farm livestock than rodents in other parts of the world where the disease is either rare or not known to occur.

SUMMARY

Aujeszky's disease virus generally produced a highly fatal disease in laboratory rats and mice. Younger mice were particularly susceptible and compared favourably with tissue culture methods for the isolation and identification of the virus.

The distribution of the virus in the body varied with the route of inoculation, but all rats and mice dying from the disease had virus in their brains. Virus was also frequently present in the kidneys of infected mice, depending upon the route of inoculation.

Adult mice occasionally recovered after showing early clinical symptoms of the disease; some withstood repeated challenge with lethal doses of the virus.

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STUDIES ON THE VIRUS OF AUJESZKY'S DISEASE
II. Pathogenicity for Chicks

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INTRODUCTION

As a general rule mammals are susceptible to natural and experimental infection with the virus of Aujeszky's disease. Little is known about the natural disease in birds and there are conflicting reports about the relative susceptibilities of different species of birds to the experimental infection.

Aujeszky (1902), Schmeidhoffer (1910) and Zwick and Zeller (1911) reported that poultry possess a slight susceptibility to the virus of Aujeszky's disease and Shope (1931), while failing to infect chickens by the subcutaneous or intratracheal routes, produced a fatal infection in 2 out of 3 birds that were inoculated intracerebrally. Jonnesco (1934) was successful in infecting pigeons and cockerels by both intracerebral and intramuscular routes and showed that the blood was infective to rabbits, while Remlinger and Bailly (1934) found that a few birds that had been successfully infected by the intracerebral route recovered from the clinical disease. This was also the experience of Gerlach and Schweinburg (1935) who inoculated the virus intracerebrally in pigeons, ducks and hens. Subsequently, Nicolau, Cruveilhier and Kopciowska (1937) reported that pigeons inoculated by the intracerebral route invariably succumbed to the disease, whereas fowls were relatively resistant. Bang (1942) noted that 2-day old chicks were susceptible in 50 per cent. of cases and Ivanovics, Abraham and Koch (1954) successfully infected chickens between 1 and 16 days of age by both the intracerebral and subcutaneous routes and recovered the virus from the brains.

Thus, the factual information on the susceptibility of chickens to the virus of Aujeszky's disease is conflicting and it is the purpose of this paper to report on the pathogenicity of the virus for chicks of different age groups.

MATERIALS AND METHODS

Virus. The virus was originally isolated in rabbit kidney cell culture from a case of Aujeszky's disease in a pig (McFerran and Dow, 1965) and was the same as that reported in a previous communication (Fraser and Ramachandran, 1969). The virus was propagated on a continuous line of pig kidney cells and, at the height of the cytopathic changes, the cell sheet was separated from the glass by gentle shaking. The cells, together with the culture fluid, were homogenized in an ultrasonic disintegrator and, after light centrifugation, the supernatant was stored in small aliquots at −65°C. The virus pools were assayed for infectivity in the same pig kidney cell line. The LD_{50} values of infectivity titres were calculated according to the method of Reed and Muench (1938) and the procedure for estimating the approximate error of LD_{50} was that of Pizzi (1950).

Tissue culture and virus isolations. The methods of propagating and maintaining the continuous line of pig kidney cells (PK15) and the procedures for virus isolations

were essentially the same as those reported previously (Fraser and Ramachandran, 1969).

Experimental chicks. These were White Leghorn chicks hatched in the laboratory from eggs obtained from the Edinburgh College of Agriculture.

TABLE 1

MORTALITY IN CHICKS LESS THAN 24 HOURS OF AGE

Route of inoculation	*Dose* (ml.)*	*Mortality*	
		No.	*%*
Intracerebral	0·05	10/10	100
Intramuscular	0·1	12/15	80
Skin scarification	0·05 −0·1	8/11	73
Subcutaneous	0·1	9/15	60
Intranasal	0·05 −0·1	7/27	26
Intraocular	0·05 −0·1	5/27	19

* $TCID_{50}$ of virus was $10^{6·3}$ per 0·2 ml.

Numerator = number dead
Denominator = number inoculated

RESULTS

The susceptibility of young chicks to infection with the virus of Aujeszky's disease was investigated in small groups of chicks of less than 24 hours of age that were infected by various routes. The virus had a titre of $10^{6·3}$ $TCID_{50}$ per 0·2 ml. The results of this preliminary experiment (Table 1) showed that day-old chicks were susceptible to the virus by different routes of inoculation and that the mortality rate was highest (100 per cent.) in birds infected intracerebrally. The mortality patterns varied in chicks infected by other routes and the virus was isolated from the brain tissues of all birds dying from the disease, irrespective of the method of inoculation.

TABLE 2

MORTALITY PATTERN IN RELATION TO AGE IN CHICKS INFECTED INTRACEREBRALLY

Age (days)	*Mortality*		*Days to death*									
	No.	*%*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
1	20/20	100	8	6	3	1	2	—	—	—	—	—
7	15/17	88	5	7	2	—	—	—	—	—	1	—
14	15/19	79	—	4	3	1	6	1	—	—	—	—
21	15/17	88	1	3	—	2	4	4	—	—	1	—
28	21/26	81	2	—	—	4	12	2	—	1	—	—
42–49	17/24	71	1	—	3	2	6	2	2	1	—	—
90–180	6/8	75	—	—	—	3	—	2	—	1	—	—

$TCID_{50}$ of virus was $10^{6·83}$ per 0·2 ml.

Numerator = number dead
Denominator = number inoculated.

Because of the high mortality rates obtained in day-old chicks infected by the intracerebral and intramuscular routes, experiments were carried out to ascertain the extent to which the susceptibility of chickens to infection with Aujeszky's disease virus might be influenced by such factors as age and dose.

Intracerebral Infection

The mortality pattern in relation to age was studied in 7 groups of chickens whose ages ranged from 1 to 180 days. The virus had a titre of $10^{6\cdot83}$ $TCID_{50}$ per 0·2 ml. and was inoculated by the intracerebral route in 0·05 to 0·1 ml. amounts. The results (Table 2) showed that the distribution of mortalities was fairly uniform in the day-old and 7 day-old groups. In chickens of the older age groups there was a prolongation of the survival period, according to age, and a reduction in the percentages of mortality. There was evidence that the delayed deaths were also associated with the infection since virus was recovered from the brains of all birds dying within the first 10 days following inoculation. Attempts to isolate virus from heart, lung, liver, spleen, kidney and spinal cord were unsuccessful.

The possibility that the nature and severity of the infection might be influenced by the dose of virus was investigated in chickens of age groups ranging from 1 to 49 days. Each bird was inoculated intracerebrally with 0·05 ml. of ten-fold dilutions of the standard cell-culture virus. It is evident from the results in Table 3

TABLE 3

MORTALITY PATTERN IN RELATION TO AGE AND DOSE IN CHICKENS INOCULATED INTRACEREBRALLY

Dilution of virus (log_{10})	*Age, in days, at time of inoculation*					
	1	*7*	*14*	*21*	*28*	*42–49*
Neat	10/10	5/5	7/8	6/7	9/13	6/10
−1	10/10	3/5	5/8	7/8	12/13	4/7
−2	9/10	4/5	7/8	5/10	10/15	4/8
−3	9/10	3/5	3/8	3/5	7/10	1/8
−4	8/10	3/5	2/8	1/5	3/10	1/8
−5	4/10	3/5	2/8	0/5	2/10	1/7
−6	7/10	0/5	0/8	—	0/5	0/5
−7	0/10	—	0/8	—	0/5	0/5
−8	—	—	0/5	—	0/5	0/5
log LD_{50} (S.E.)	−5·12 (±0·401)	−3·78 (±0·647)	−2·73 (±0·430)	−2·24 (±0·455)	−2·91 (±0·393)	−1·50 (±0·472)

$TCID_{50}$ of virus was $10^{6\cdot83}$ per 0·2 ml.

Numerator = number dead
Denominator = number inoculated

that the virus of Aujeszky's disease was pathogenic for both the younger and older groups of chickens and that the mortality from experimental infection declined progressively with age. The difference between the LD_{50} values for day-old and 7-day old birds (5·12 ± 0·401 and 3·78 ± 0·647; $P<0·01$) and for 7-day old and 42 to 49-day old birds (3·78 ± 0·647 and 1·50 ± 0·472; $P<0·01$) are statistically significant. The results show that the mortality is also related to dose.

Intramuscular Infection

Chickens of different ages ranging from less than 6 hours to 42 days were inoculated into the muscles of the thigh. Very young chickens were inoculated with 0·1 ml. whereas birds over 7 days of age each received 0·2 to 0·5 ml. of virus. A lethal effect occurred only in chickens under 48 hours of age (Table 4). There were no adverse effects in older age groups, despite the fact that some were inoculated with larger amounts of virus. In fatal infections virus was recovered from the brain as well as from the site of inoculation and the spinal cord, but not from the heart, lungs, spleen, liver and kidneys.

The effect of the dose of the virus was investigated further by inoculating chickens ranging in age from less than 12 hours to 72 hours or older, with serial ten-fold dilutions of the virus. The results of this experiment (Table 5) confirmed the previous finding that intramuscular inoculation had no adverse effects in chicks of 48 hours of age or older. The mortality in chicks under 48 hours of age was related to the dose of the virus.

TABLE 4

MORTALITY PATTERN IN RELATION TO AGE IN CHICKENS INFECTED INTRAMUSCULARLY

Dose of virus (ml.)	*Age*	*Mortality*		*Days to death*									
		No.	*%*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
0·1	1–6 hr	9/9	100	—	—	5	3	—	—	—	—	1	—
0·1	1–12 hr	11/11	100	—	2	9	—	—	—	—	—	—	—
0·1	1–18 hr	6/7	86	—	—	3	2	1	—	—	—	—	—
0·1	1–24 hr	21/30	70	1	—	7	8	—	2	—	—	3	—
0·1	24–48 hr	4/15	27	—	—	2	1	1	—	—	—	—	—
0·1	48–66 hr	0/3	0	—	—	—	—	—	—	—	—	—	—
0·1	72 hr	0/18	0	—	—	—	—	—	—	—	—	—	—

$TCID_{50}$ of virus was $10^{6\cdot3}$ per 0·2 ml.

Numerator = number dead
Denominator = number inoculated

No adverse effects were observed when 34 chicks between 4–42 days of age were inoculated with 0·1 to 0·5 ml. of undiluted virus.

Clinical Syndrome

Chicks of up to 24 hours of age infected by different routes developed no distinctive clinical features except those that are commonly associated with nervous injury. However, general malaise, anorexia, drowsiness, gasping, muscular tremors and incoordination of movement were prominent in most fatal cases irrespective of age. Another common feature was the escape of stringy saliva from the beaks of chicks in the terminal stages of the infection. Paresis of the inoculated leg was invariably associated with intramuscular infection in chicks of up to 48 hours of age. Hyperaesthesia of the skin, a dominant feature in the symptomatology of the experimental disease in rabbits, rats and mice, did not occur.

TABLE 5

MORTALITY PATTERN IN RELATION TO AGE AND DOSE IN CHICKS INOCULATED INTRAMUSCULARLY

Dilution of virus (log_{10})	*Age (hours) at inoculation*					
	1–12	*1–18*	*1–24*	*24–48*	*48–66*	*>72*
Neat	20/20	6/7	21/30	4/15	0/3	0/4
−1	12/14	7/8	5/21	0/7	0/3	0/4
−2	9/14	6/8	4/21	3/7	0/3	0/4
−3	4/8	1/8	1/15	1/7	0/3	0/4
−4	0/8	0/6	0/13	0/7	0/3	0/4
−5	0/7	0/6	0/10	0/5	0/3	0/4
−6	0/3	0/3	0/10	0/5	—	0/4
−7	0/3	—	0/10	0/2	—	0/4
−8	0/3	—	0/10	0/2	—	0/4
log LD_{50} (S.E.)	−2·39 (±0·295)	−2·25 (±0·362)	−0·57 (±0·214)	—	—	—

$TCID_{50}$ of virus was $10^{6·3}$ per 0·2 ml.

Numerator = number dead
Denominator = number inoculated

Resistance to Infection

The results of the above experiments showed that there was a gradual extension of the survival period and a reduction in the percentages of mortality in infected chickens of the higher age groups. The nature of this age associated resistance was investigated further by challenging those chickens that had survived from the earlier experiments shown in Table 3, by inoculating them intracerebrally with

TABLE 6

RESISTANCE TO REINFECTION IN CHICKENS OF DIFFERENT AGES

Dilution of virus in primary inoculum (log_{10})	*Age at primary intracerebral inoculation (P/i.c) and at challenge intracerebral inoculation (C/i.c)*					
	1–7 days *P* *i.c*	*3–6 weeks* *C* *i.c*	*14–21 days* *P* *i.c*	*4–9 weeks* *C* *i.c*	*28–49 days* *P* *i.c*	*6–13 weeks* *C* *i.c*
Neat	15/15	—	13/15	0/2	15/23	0/7
−1	13/15	0/2	12/16	0/4	16/20	0/3
−2	13/15	0/2	12/18	0/5	14/23	0/6
−3	12/15	0/3	6/13	0/5	8/18	0/7
−4	11/15	0/4	3/13	2/6	4/18	2/10
−5	7/15	0/7	2/13	6/9	3/17	4/11
−6	7/15	5/8	0/8	7/8	0/10	2/8
−7	0/10	6/9	0/8	5/6	0/10	5/8
−8	—	—	0/5	2/4	0/10	5/7
Controls	19/22		21/27		35/53	

Numerator = number dead
Denominator = number inoculated

All chicks surviving the primary inoculation, except for a few that died from non-specific causes, were challenged with undiluted virus.

$TCID_{50}$ of virus was $10^{6·83}$/0·2 ml.

undiluted virus. A number of control birds of corresponding age were similarly infected. Analysis of the data from this experiment (Table 6) indicates that the ability of chickens to resist reinfection cannot be explained entirely in terms of age-associated tolerance, but is largely influenced by the amount of virus in the primary inoculation. In general, chickens that received primary inocula consisting of virus dilutions up to 10^{-4} withstood a subsequent challenge dose of undiluted virus given several weeks later, whereas most of the controls of the same age succumbed to the infection. The overall mortality in chickens that received higher dilutions of virus in the primary inoculum, and were subsequently challenged with undiluted virus, was similar to that of the challenge control group. Resistance was pronounced, especially in day-old and 7-day old chicks.

The nature of the resistance to intracerebral challenge was likewise investigated in groups of chicks that had survived intramuscular infection (Table 5) with different doses of the virus. As before, it was found that the resistance of chicks to challenge infection was related to the dose of the virus in the primary inoculum (Table 7), and dilutions up to 10^{-4} were sufficient to initiate the state of resistance. It is probable that the resistance to challenge infection in chicks of the 48 to 66 hours age group is induced by the primary inoculation and is not an age-associated phenomenon. At the time of the challenge infection the chicks in this group were 21 days old, an age at which susceptibility to intracerebral inoculation is usual.

TABLE 7

RESISTANCE TO REINFECTION IN CHICKENS OF DIFFERENT AGES

Dilution of virus in primary inoculum (log_{10})	*Age at primary intramuscular inoculation (P/i.m.) and at challenge intracerebral inoculation (C/i.c.)*					
	<24 hours *P* *i.m.*	*2–3 weeks* *C* *i.c.*	*24–48 hours* *P* *i.m.*	*3 weeks* *C* *i.c.*	*2–9 days* *P* *i.m.*	*3–4 weeks* *C* *i.c.*
Neat	47/57	0/7	4/15	0/9	0/7	0/5
−1	24/43	0/18	0/7	0/7	0/7	0/6
−2	19/43	1/21	3/7	0/4	0/7	1/7
−3	6/31	1/20	1/7	0/6	0/7	3/7
−4	0/27	1/23	0/7	0/7	0/7	0/6
−5	0/23	3/18	0/5	1/4	0/7	3/7
−6	0/16	5/14	0/5	2/5	0/4	4/4
−7	0/13	7/12	0/2	2/2	0/4	2/4
−8	0/13	7/13	0/2	1/1	0/4	3/4
Controls	55/73		9/10		7/8	

Numerator = number dead
Denominator = number inoculated

All chicks surviving the primary intramuscular inoculation, except for a few that died from non-specific causes, were reinfected intracerebrally with undiluted virus.

$TCID_{50}$ of virus was $10^{6 \cdot 83}$/0·2 ml.

DISCUSSION

The literature contains evidence that poultry may possess a slight susceptibility to the virus of Aujeszky's disease, but most of the published experiments are not of a quantitative nature

Fraser and Ramachandran (1969) reported that the infectivity titres in experimental Aujeszky's disease of day-old and week-old mice were comparable to the titres generally obtained in a pig kidney cell line and the present findings have established that day-old chicks are also highly susceptible. The LD_{50} titres obtained by intracerebral inoculation were similar to those in adult mice and rats.

The mortality, duration and clinical signs of the disease in young chicks were reminiscent of the disease in mice and rats except that hyperaesthesia of the skin which is a dominant feature of the disease in rats and mice (Fraser and Ramachandran, 1969), as it is in other animals (Galloway, 1938) was absent. Moreover, in intracerebral infection, as in the rodent disease, the virus was recovered from the brains of chicks, but not from the other tissues examined.

Much of the confusion in the early literature on the susceptibility of poultry to experimental infection stems from the fact that birds of undetermined and possibly older age-groups were usually used. The present findings established the decisive influence of age on the susceptibility of the chicken to fatal infection with Aujeszky's virus. In groups of chicks infected intracerebrally a highly significant, inverse relationship was observed between the age of the chicks and the percentage mortality. With increasing age the percentage of deaths dropped progressively and there was also an extension of the mean time to death. Bang (1942) found that the severity of lesions in the embryonating hen's egg decreased with the increasing age of the embryo.

The effect of age on the susceptibility of birds was particularly striking when the virus was administered by the intramuscular route. Amounts of virus lethal for chicks between 6 and 36 hours of age failed completely to produce mortality in birds of 48 hours of age or older. Virus could be isolated from the inoculation site, spinal cord and brain of day old chicks which died after intramuscular inoculation. Failure to recover virus from heart, lung, liver, spleen and kidney suggest that these sites are unfavourable for replication. Alternatively, the virus may be present in different tissues in low, undetectable levels, or intracellularly as latent virus.

The mortality in chickens was found to be dose-dependent, irrespective of age or route of inoculation.

Many of the chicks that survived were resistant to reinfection. The ability to withstand reinfection even by the intracerebral route in very young chickens was clearly related to the dose of virus in the first inoculum. Survival of chicks of older age-groups following reinfection was likewise influenced by the primary inoculation, and was independent of age-associated resistance to fatal infection, since the mortality was markedly higher in groups of control chicks of corresponding age.

These findings show that susceptibility to experimental infection with this particular strain of virus is influenced by the age of the chicken, the dose of the virus and the mode of infection. Little is known about the natural disease in

domestic poultry, even in endemic areas, and it is possible that young chicks may acquire the infection without showing the classical symptoms of the disease.

SUMMARY

Chicks were highly susceptible to experimental infection with Aujeszky's disease virus by different routes during the first 24 hours after hatching. Intracerebral inoculation produced 100 per cent. deaths. In higher age groups, the mortality rates following intracerebral infection were related to the age of the chicks and the dose of the virus. Intramuscular inoculation failed to induce mortality in chicks over 48 hrs. of age. Virus was recovered from the brains of chicks that succumbed to the disease, irrespective of the route of infection. Chicks that survived intracerebral or intramuscular inoculation were resistant to reinfection with higher doses of the virus.

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